molecules existing in the rigid, gel configuration. The observed increase in the main transition temperature of DPPC could reflect a better solubility of glucocerebroside in the gel phase than in the liquid crystalline phase of DPPC. If this is so, the formation of pure glucocerebroside domains is more likely to occur in the liquid crystalline phase. Thus, the gel-to-liquid transition may be accompanied by an increase in the average size of glucocerebroside domains. At the present time it is not clear whether the molecular origin of the above effects is due to head-group interactions or fatty acyl chain interactions or a combination of both.

From the point of view of membrane function, the possibility that the in-plane distribution of glucocerebroside molecules might be modulated by the physical state of the lipid bilayer is very interesting and is currently under study in this laboratory. Techniques such as freeze-fracture electron microscopy are being utilized for obtaining independent quantitative assessments of this distribution.

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# Gross Structural Changes in Isolated Liver Cell Plasma Membranes upon Binding of Insulin†

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ABSTRACT: The addition of $10^{-9}$ M insulin to a suspension of rat liver plasma membranes increases the overall lipid microviscosity, $\bar{\eta}$, by about 10–20%. The effect is confined to physiological concentrations of the hormone and is highly specific. The specificity was demonstrated in experiments where insulin analogues were added to liver plasma membranes and where insulin was added to human erythrocyte membranes. In both of these experiments practically no change in $\bar{\eta}$ was detected. Upon in vitro enrichment of the membrane cholesterol, $\bar{\eta}$ exceeded the level mediated by insulin binding, and the addition of $10^{-9}$ M insulin to the cholesterol-enriched membranes did not further increase $\bar{\eta}$. Concomitant to the increase in $\bar{\eta}$ upon insulin binding, the overall degree of exposure of the membrane protein, presumably to both sides of the membrane, is substantially increased. This effect is in line with the notion of vertical displacement of membrane proteins induced by changes in $\bar{\eta}$. The observed structural modulation can account for the effect of insulin on unrelated membrane responses, as well as for the negative cooperativity of insulin binding.

The concept that the mechanism of action of insulin is mediated through an interaction with the plasma membrane of the target cell has played a pivotal role in the studies on the physiological effects of the hormone (Stadie, 1954; Levine & Goldstein, 1955; Pilkis & Park, 1974; Steiner, 1977). The relevance of the hormone–membrane interaction has received further support in recent years from the extensive studies carried out on the membrane-bound receptor for insulin (Cuatrecasas, 1973; Kahn, 1976; Ginsberg, 1977). Attempts to relate the manifold action of the hormone to the general hypothesis of cAMP serving as the second messenger for the information carried by the hormone molecule (Robison et al., 1971) did not yield unequivocal results (Goldfine, 1977). In addition, evidence has been reported in recent years as to the presence of intracellular binding sites for insulin which would explain its intracellular effects which follow internalization (Goldfine, 1977; Steiner, 1977). In such a complex framework

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of action only the interaction of insulin with the plasma membrane is universally accepted as the very first step of the action of the hormone. The overt physiological responses, which involve membrane-linked processes or intracellular biochemical events, follow this step.

Recently, it has been suggested that a general feature of the mechanism of action of insulin at the plasma membrane level involves a decrease in fluidity of the lipid layer. This suggestion was inferred from studies with cells not known to be physiological targets for insulin such as mammalian red cells (Massa et al., 1975) and bacterial membranes (Moreno & Farias, 1976), where the effect of insulin binding on the cooperative behavior of membrane-bound enzymes (Farias et al., 1975) was determined. However, a similar effect of insulin was also observed in isolated liver plasma membranes (Luly et al., manuscript in preparation).

The following study demonstrates that the binding of insulin to liver plasma membranes, which is very rich in insulin receptor (Ginsberg, 1977), is accompanied by a 10–20% increase in the overall lipid microviscosity. This increase mediates a nonspecific increase of the degree of exposure of the membrane proteins which could, in turn, modulate the response to insulin and a series of unrelated membrane activities.

## Materials and Methods

Porcine insulin, proinsulin, diarginine insulin, desalanine insulin, and desoctapeptide insulin were kindly supplied by Dr. R. E. Chance, Lilly Research Laboratories, Indianapolis, IN. Trypsin was the once crystallized salt-free product from Worthington. *N*-Methylpicolinium perchlorate was prepared as previously described (Shinitzky & Rivnay, 1977).

Livers from male Wistar rats, 180-g average body weight, fed ad libitum and having free access to water, were used in this study. Liver plasma membranes were isolated following the Ray (1970) technique as reported previously (Luly & Verna, 1974; Tria et al., 1977) and finally resuspended in Tris-buffered saline (0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl) at an average protein concentration of 0.7 mg/mL. Membrane purity was established as described by Luly et al. (1972). The method of Lowry et al. (1951) was followed to estimate protein concentration using bovine serum albumin as a standard.

Liver plasma membrane microviscosity was determined with the probe 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] using the instrumentation and method which were described previously (Shinitzky & Inbar, 1976; for a review see Shinitzky & Barenholz, 1978). A solution of $2 \times 10^{-3}$ M DPH in tetrahydrofuran was first dispersed by 1000-fold agitative dilution in Tris-buffered saline. Membrane suspensions were mixed 1:1 with the probe dispersion, followed by a 15 min incubation at 37 °C at a protein concentration of 50 μg/mL. During this incubation insulin and its analogues were also present at the concentration indicated. The degree of fluorescence polarization, $P$, of the labeled membranes was measured, and the corresponding microviscosity, $\bar{\eta}$, was determined as described (Shinitzky & Inbar, 1976; Shinitzky & Barenholz, 1978). The temperature of the samples was controlled with a circulation bath and measured directly in the sample with a thermistor of an accuracy of 0.01 °C. Excited-state lifetime, $\tau$, was measured at 25 °C by a pulse sampling technique, followed by a deconvolution analysis (Grinvald & Steinberg, 1974). For other temperatures $\tau$ was estimated from the relative fluorescence intensity (Shinitzky & Barenholz, 1974).

[1] Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; C/PL, cholesterol/phospholipid (M/M).

Table I: Insulin Effect on the Microviscosity of Liver Plasma Membranes at 37 °C[a]

| | $P$ at 37 °C | | $\bar{\eta}$ (P) at 37 °C | | |
|---|---|---|---|---|---|
| expt | −insulin | +insulin | −insulin | +insulin | $\Delta\bar{\eta}$ (%) |
| 1 | 0.263 | 0.289 | 2.77 | 3.53 | 27 |
| 2 | 0.279 | 0.295 | 3.25 | 3.73 | 15 |
| 3 | 0.250 | 0.270 | 2.42 | 2.95 | 22 |
| 4 | 0.235 | 0.242 | 2.12 | 2.27 | 7 |
| 5 | 0.243 | 0.253 | 2.27 | 2.49 | 10 |
| 6 | 0.260 | 0.272 | 2.69 | 2.95 | 10 |
| 7 | 0.261 | 0.273 | 2.72 | 3.01 | 11 |

[a] Plasma membranes from individual rats were incubated for 15 min at 37 °C with or without $10^{-9}$ M porcine insulin in the presence of $2 \times 10^{-6}$ M DPH dispersion. The excited-state lifetimes, $\tau$, in the absence and presence of insulin were 8.8 ± 0.1 and 9.0 ± 0.1 ns. With the aid of these values and the determined $P$ values the given microviscosities $\bar{\eta}$ were evaluated.

The degree of exposure of membrane proteins was determined by quenching of tryptophan fluorescence as described by Shinitzky & Rivnay (1977). For this measurement an Aminco-Bowman spectrofluorimeter was used, with an excitation at 290 nm and emission at 340 nm. Suspensions of membranes at a concentration of 60 μg of protein/mL in Tris-buffered saline were measured after 15 min of incubation with or without insulin. All measurements were taken at 37 °C in the presence of *N*-methylpicolinium perchlorate as a quencher and tetramethylammonium perchlorate as a control. Total availability of membrane tryptophans to quenching was estimated by mixing one volume of membrane suspension (60 μg/mL) in Tris-buffered saline with two volumes of 2,2,2-trifluoroethanol (Merck Uvasol) (Shinitzky & Rivnay, 1977).

The cholesterol/phospholipid molar ratio (C/PL) of isolated liver plasma membranes was altered in vitro following a procedure which was recently described (Shinitzky, 1978). Briefly, one volume of liver membranes suspension (0.5–1 mg of protein/mL) was incubated for 6 h at 37 °C with two volumes of heat-inactivated 10% fetal calf serum in Dulbecco-modified Eagle's medium supplemented with 0.1 g/L of dihydrostreptomycin sulfate and sodium penicillin G, 100 000 units/L. The cholesterol-enriching medium contained an additional 0.13 mg/L of cholesterol, whereas cholesterol-depleting medium contained an additional 0.25 mg/L of egg lecithin. After incubation, the plasma membranes were washed four times with Tris-buffered saline and resuspended in the same buffer at the initial protein concentration. Cholesterol was determined as total cholesterol according to Chaimori & Henry (1959). Phospholipid contents were determined by a modification of the Fiske–SubbaRow procedure (Bottcher et al., 1961).

## Results

The different preparations of liver plasma membranes which were studied revealed considerable variability in the apparent degree of fluorescence polarization, $P$, after labeling with DPH. However, in all membrane samples the $P$ value was found to increase by about 10–20% upon treatment with insulin. Thus, the lipid microviscosity, $\bar{\eta}$, of the liver plasma membrane can vary between individual rats, but the extent of the effect of insulin binding remains basically unaltered ($p < 0.1$). Table I presents the values of $\bar{\eta}$ of seven different preparations of rat liver plasma membranes and the effect of $10^{-9}$ M insulin on it. The results strongly suggest that in the range of physiological concentrations (Illiano & Cuatrecasas, 1972) the hormone acts to increase the overall lipid microviscosity of the liver cell plasma membranes. The apparent effect of $10^{-9}$ M insulin on the $P$ value of DPH and the corresponding

Table II: Insulin Effect on the Microviscosity of Liver Plasma Membranes at Various Temperatures[a]

| temp (°C) | no. of expt | −insulin $\bar{\eta}$ (P) | −insulin $P$ | −insulin $\tau$ (ns) | +insulin $\bar{\eta}$ (P) | +insulin $P$ | +insulin $\tau$ (ns) |
|---|---|---|---|---|---|---|---|
| 4 | 4 | 11.25 ± 1.81 | 0.376 | 11.2 | 13.04 ± 2.21 | 0.381 | 11.3 |
| 12 | 4 | 8.11 ± 0.68 | 0.350 | 10.7 | 9.14 ± 1.00 | 0.360 | 10.9 |
| 25 | 4 | 4.32 ± 0.32 | 0.302 | 9.2 | 4.73 ± 0.41 | 0.312 | 9.5 |
| 37 | 7 | 2.60 ± 0.14 | 0.256 | 8.8 | 2.99 ± 0.23 | 0.270 | 9.0 |

[a] Results are reported as the mean microviscosity ($\bar{\eta}$ ± SE) and as the corresponding average $P$ and $\tau$ values.

Table III: Dose Response of the Effect of Insulin on Liver Plasma Membrane Microviscosity[a]

| insulin concn (M) | $\bar{\eta}/\bar{\eta}_0$ | $P$ |
|---|---|---|
| 0[b] | 1.000 | 0.243, 0.261, 0.250 |
| $1.10^{-11}$ | 1.015 (1.010–1.020) | 0.244, 0.263, 0.253 |
| $1.10^{-10}$ | 1.030 (–) | 0.245, 0.265, 0.255 |
| $1.10^{-9}$ | 1.100 (–) | 0.251, 0.271, 0.260 |
| $1.10^{-8}$ | 1.115 (1.110–1.120) | 0.254, 0.272, 0.268 |
| $1.10^{-7}$ | 1.100 (1.090–1.110) | 0.254, 0.270, 0.269 |
| $1.10^{-6}$ | 1.100 (1.090–1.110) | 0.252, 0.270, 0.268 |

[a] Plasma membranes were preincubated for 15 min at 37 °C with or without the hormone at the concentration indicated. Microviscosity, $\bar{\eta}$, is reported as the ratio of the observed value of $\bar{\eta}$ at 37 °C with respect to control ($\bar{\eta}_0$). Results are means of three separate experiments carried out on different membrane preparations. The $P$ values of each experiment are reported. The determined $\tau$ values were all in the range of 8.7–9.1 ns (see Table I). [b] Control.

microviscosity at different temperatures is given in Table II. It should be noted that when insulin was added to membranes which were prelabeled with DPH the increase in $P$ value at both 4 and 37 °C was within less than 5 min. The kinetics of the effect, however, has not been studied any further.

The dose response of the insulin effect was tested at a wide range of hormone concentration (Table III), and the results indicate that the sensitivity of the plasma membrane microviscosity to the presence of insulin is confined to physiological concentrations. Furthermore, the tested insulin concentrations (Table III) should be considered as upper limits since the degradation of the hormone by the membrane enzymes is rather efficient (Freychet et al., 1972). The specificity of the insulin effect on liver plasma membrane microviscosity was also examined with proinsulin and insulin congeners (see Table IV). The results obtained show a correlation between the effect of proinsulin and insulin on the membrane lipid fluidity and their known biological potency (Steiner et al., 1969; Blundell et al., 1972). Only desalanine and diarginine insulin showed some effect, which is in agreement with studies on liver plasma membrane bound cAMP phosphodiesterase sensitivity to insulin (Tria et al., 1977). Table IV also includes results of experiments concerning trypsin effect on plasma membrane microviscosity. These experiments were performed in order to rule out any possible linkage between the observed insulin effect and the alleged trypsin-like activity of the hormone (Huang & Cuatrecasas, 1975). The lack of any effect of trypsin is in agreement with its uneffectiveness on plasma membrane microviscosity (Rudy & Gitler, 1972), as well as on membrane-bound cAMP phosphodiesterase (Tria et al., 1977).

The temperature dependence of the microviscosity of isolated rat liver plasma membranes was determined in four samples after preincubation with or without $10^{-9}$ M insulin. Simultaneous determinations of $P$ and $\tau$ in the range of 3–40

Table IV: Effect of Insulin, Proinsulin, Insulin Cogeners, and Trypsin on the Microviscosity of Liver Plasma Membranes[a]

| | | $\bar{\eta}/\bar{\eta}_0$ | $P$ |
|---|---|---|---|
| control | | 1.000 | 0.243, 0.261, 0.250 |
| insulin | $1.10^{-9}$ M | 1.110 (–) | 0.253, 0.271, 0.260 |
| proinsulin | $1.10^{-9}$ M | 1.011 (0.993–1.030) | 0.245, 0.261, 0.252 |
| desalamine insulin | $1.10^{-9}$ M | 1.022 (–) | 0.244, 0.261, 0.251 |
| desoctapeptide insulin | $1.10^{-9}$ M | 0.991 (0.982–1.000) | 0.243, 0.259, 0.249 |
| diarginine insulin | $1.10^{-9}$ M | 1.020 (1.011–1.030) | 0.245, 0.261, 0.249 |
| trypsin | 1 μg/mL | 1.001 (0.993–1.009) | 0.243, 0.261, 0.249 |

[a] Plasma membranes were preincubated for 15 min at 37 °C with or without the substances under investigation. Microviscosity is reported as the ratio of the observed value of $\bar{\eta}$ at 37 °C with respect to control. Results are means of three experiments carried out on different membrane preparations. The $P$ values of each experiment are reported. The determined $\tau$ values were all in the range of 8.9 ± 0.1 ns (see Table I).

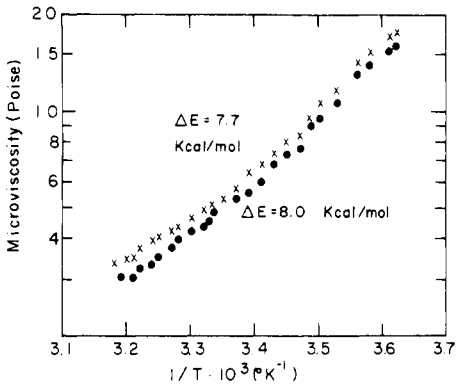


FIGURE 1: Temperature dependence of the lipid microviscosity of rat liver plasma membranes before (●) and after (X) treatment with $1.10^{-9}$ M insulin.

°C were carried out at temperature intervals of 2–3 °C. In each of the samples and at all temperatures $\tau$ never exceeded the upper level of $\tau_0$ = 11.4 (Shinitzky & Barenholz, 1974) and $P$ was consistently greater in the presence of insulin. The results of all samples displayed an approximate linear dependence of log $\bar{\eta}$ on $1/T$ (Shinitzky & Inbar, 1976) with a slightly steeper slope (greater flow activation energy, $\Delta E$) in the absence of insulin. The profiles obtained with one membrane preparation are shown in Figure 1. From the slope of the best linear fit of the plots given in Figure 1 the derived $\Delta E$ value for insulin-treated membranes was 7.7 kcal/mol and 8.0 kcal/mol for untreated membranes.

The well-established dependence of membrane microviscosity parameters on C/PL ratio (Shinitzky & Inbar, 1976; Cooper et al., 1978) prompted us to change in vitro this ratio following a new method which employs conditions close to physiological (Shinitzky, 1978). Results given in Table V show that the observed effect of insulin on liver membrane microviscosity disappears when the membrane itself has an increased microviscosity due to cholesterol enrichment. The C/PL ratio observed in the present research for untreated liver plasma membranes (see footnote *a*, Table V) is in good agreement with published data (Emmelot & van Hoeven, 1975; Collard et al., 1977). It should be noted that in the control treatment reported in Table V both the C/PL ratio and the microviscosity are higher than those reported in Table II for the same temperature. This increase can be attributed

Table V: Effect of Insulin on the Microviscosity of Liver Plasma Membranes with an Altered Cholesterol/Phospholipid Mol Ratio[a]

| treatment | −insulin $\bar{\eta}$ | −insulin $P$ | +insulin $\bar{\eta}$ | +insulin $P$ | C/PL (mol/mol) |
|---|---|---|---|---|---|
| control | 3.14 ± 0.22 | 0.275 | 3.52 ± 0.11 | 0.286 | 0.75 |
| cholesterol depletion | 2.87 ± 0.07 | 0.266 | 3.19 ± 0.27 | 0.276 | 0.71 |
| cholesterol enrichment | 3.78 ± 0.18 | 0.294 | 3.72 ± 0.16 | 0.292 | 1.03 |

[a] Plasma membranes, treated as described in the text, were preincubated for 15 min at 37 °C with or without $1 \times 10^{-9}$ M insulin. The results are expressed as $\bar{\eta}$ ± SE and are means of four separate experiments carried out on different membrane preparations; average $P$ values are also reported. C/PL ratio values are means of duplicate experiments. The C/PL ratio determined for untreated liver plasma membranes was 0.69, which represents a mean of two determinations carried out on different membranes preparations.

to the high C/PL molar ratio in fetal calf serum as compared to rat serum (Long, 1961). It should be stressed, however, that insulin effect on the lipid microviscosity remained approximately unaltered. Experiments analogous to those reported in Table V were carried out on human erythrocyte ghost membranes. The results obtained (not shown) agreed well with previously published data (Cooper et al., 1978; Shinitzky, 1978) but did not display any insulin effect. This rules out the possibility of nonspecific effect of the hormone on membrane fluidity parameters (Massa et al., 1975; Moreno & Farias, 1976).

The effect of insulin on the degree of exposure of proteins in the liver plasma membranes was examined by fluorescence quenching (Shinitzky & Rivnay, 1977). Collisional quenching of fluorescence in a homogeneous system obeys the Stern–Volmer equation (Stern & Volmer, 1919)

$$F_0/F = 1 + K_q[Q] \quad (1)$$

where $F_0$ and $F$ are the fluorescence intensities in the absence and presence of a quencher where concentration is indicated by [Q]. $K_q$ is the quenching constant which is the product of a collisional rate constant and the fluorescence lifetime of the fluorophore. In a mixed population of fluorophores, like the tryptophan residues of a protein in solution, eq 1 will expand into a series which was given by Lehrer (1971)

$$\frac{F_0}{F_0 - F} = \left( \sum_i \frac{f_i K_{qi}[Q]}{1 + K_{qi}[Q]} \right)^{-1} \quad (2)$$

where $f_i$ is the fraction of the total fluorescence contributed by the group $i$ in the absence of quencher.

To a good approximation, eq 2 can be simulated into a binary system in which $f$ is the fraction of the total fluorescence available for quenching, and $(1 - f)$ is the fraction which is unavailable for quenching (Lehrer, 1971). In such a system a plot of $F_0/F$ vs. [Q] should yield a normal saturation curve with a plateau at $(1 - f)^{-1}$. For convenience, eq 2 can be converted to the linear form (Lehrer, 1971)

$$\frac{F_0}{F_0 - F} = \left( \frac{1}{fK_q} \right) \left( \frac{1}{[Q]} \right) + \frac{1}{f} \quad (3)$$

This linear relation was found to express well collisional quenching of protein fluorescence in solution (Lehrer, 1971) as well as of erythrocyte membrane proteins in suspension (Borochov & Shinitzky, 1976; Shinitzky & Rivnay, 1977).

The collisional quenching profiles of the fluorescence of the liver plasma membrane proteins are shown in Figure 2.

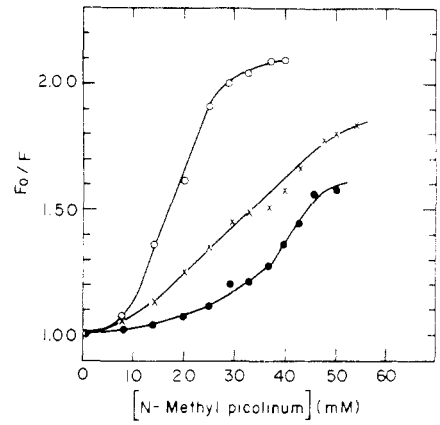


FIGURE 2: Quenching of tryptophan fluorescence of rat liver plasma membranes (60 μg of protein/mL) by *N*-methylpicolinium perchlorate in Tris-buffered saline, pH 7.5 at 37 °C. The samples measured were intact membranes (●), membranes in the presence of $10^{-9}$ M insulin (X), and membranes disintegrated with 66% 2,2,2-trifluoroethanol (○). $F_0$ and $F$ are the fluorescence intensities in the absence and presence of the quencher.

Qualitatively, the figure indicates that the binding of insulin (which does not contain tryptophans) increases the accessibility of the tryptophan residues to quenching to an intermediate level between the intact and the fully disintegrated membranes. Since the membrane preparations used here are a mixed population of vesicles and sheet-like structures (Ray, 1970; Wallach & Lin, 1973), the accessibility is presumably increased to both sides of the membrane (Borochov et al., 1978). Quantitatively, the sigmoid quenching profiles do not obey the linearity expressed in eq 3 and indicate that ground-state complexes of picolinium and tryptophan (Cilento & Giusti, 1959) contribute substantially to the quenching. In a homogeneous system such ground-state complexes will modify eq 1 to (Weller, 1959; Vaughan & Weber, 1970)

$$F_0/F = (1 + K_q[Q])(1 + K_a[Q]) \quad (4)$$

where $K_a$ is the association constant between the quencher and the fluorophore. Introduction of ground-state association into the binary system which is described above yields

$$\frac{F_0}{F} = \frac{(1 + K_q[Q])(1 + K_a[Q])}{1 + (1 - f)[(K_q + K_a)[Q] + K_q K_a[Q]^2]} \quad (5)$$

which represents a sigmoid relation between $F_0/F$ and [Q] similar to those shown in Figure 2.

## Discussion

The results reported in this study strongly support the hypothesis that the action mechanism of insulin involves modulation of the lipid fluidity of the plasma membrane. Insulin is shown to act under physiological conditions to increase the overall microviscosity of the plasma membrane by about 10–20%. The specificity of this effect is supported by our experiments with the insulin analogues. The leveling of the increase in the membrane microviscosity at insulin concentration above $10^{-9}$ M suggests that this effect is confined to the range of physiological concentrations, showing high affinity and low capacity. If one considers that the liver cell plasma membrane contains about 250 000 insulin receptors which are of high molecular weight (Ginsberg, 1977), then conformational changes induced by insulin binding (Ginsberg et al., 1976) can plausibly exert changes in the fluidity of the surrounding lipids. Insulin-induced changes in plasma membrane conformation was recently proposed by Steiner (1977) in a receptor–transducer internalization model which

could be accounted for by a shift in the plasma membrane organization toward a more ordered conformation, as is also suggested by the observed decrease of flow activation energy (Shinitzky & Inbar, 1976) in the hormone-treated membranes (see Figure 1 and Tables I and II).

The role of lipid microviscosity in regulation of the plasma membrane physiological as well as pathological behavior has obtained increasing attention in recent years (Shinitzky, 1976; Cooper, 1977). In this connection the C/PL ratio together with the degree of unsaturation of phospholipids in plasma membranes appears to play a major role in modulating membrane fluidity, as well as the activity of membrane-bound enzymes (Emmelot & van Hoeven, 1975; Brivio-Haugland et al., 1976). Therefore, the possibility of modulating plasma membrane microviscosity by altering the C/PL ratio in vitro with a simple technique (Shinitzky, 1978) offered us a useful tool to demonstrate the consistency of the reported effect of insulin on liver plasma membrane; the hormone is not effective in increasing plasma membrane microviscosity when this parameter has been already increased well above its physiological level. Thus, the hormonal effect on membrane fluidity is presumably confined to fluidities below a certain level of the C/PL ratio.

The fluorescence quenching technique could give some qualitative information as to the dynamic behavior of membrane proteins with minimum perturbations in the membrane environment. The fraction of the total tryptophan fluorescence available for quenching with *N*-methylpicolinium was found to increase in the presence of insulin to an intermediate level between the proteins in the intact membrane and the proteins in the disintegrated membranes. This effect could be accounted for, to a major extent, by an overall increase in the degree of exposure of the membrane proteins to both sides of the membrane due to the increase in lipid microviscosity which is mediated by the binding of the hormone to its receptor (Borochov & Shinitzky, 1976). In addition, local conformational changes in the receptor–hormone complex or in neighboring proteins could also contribute to the observed increase in the availability of tryptophans to the quencher.

The proposed mediation of protein displacement by the insulin–receptor interaction can passively modulate a series of unrelated membrane responses. An interesting possibility is passive modulation of the unoccupied insulin receptor itself which can account for the well-established negative cooperativity of insulin binding to target cells (Ginsberg, 1977). Preliminary observations have indicated that enrichment of turkey erythrocytes with cholesterol decreases the number of available receptors for [$^{125}$I]insulin, whereas depletion of cholesterol resulted in the converse effect (manuscript in preparation). According to the mechanism of vertical displacement of membrane proteins (Borochov & Shinitzky, 1976) only cross-membrane proteins with a substantially greater mass at the inner side than at the outer side of the membrane will be displaced toward the inside upon increase in membrane microviscosity. This, therefore, suggests that the insulin receptor, which is thought to be an asymmetric integral protein (Cuatrecasas, 1973), presumably has a bulky mass which faces the cytoplasm. Thus, the increase in membrane microviscosity, which is mediated by the binding of insulin, will follow a decrease in the number of binding sites available on the exofacial side of the membrane, which in the bulk sense will display a negative cooperativity of binding.

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# Stereochemistry of Internucleotide Bond Formation by Polynucleotide Phosphorylase from *Micrococcus luteus*†

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ABSTRACT: Polynucleotide phosphorylase catalyzes the formation of polynucleotides from the *Sp* diastereomer of adenosine 5′-*O*-(1-thiodiphosphate) (ADPαS), whereas the *Rp* diastereomer is a competitive inhibitor. The absolute configuration of the phosphorothioate diester bond in the polymer was determined by copolymerizing ADPαS,*Sp* isomer with UDP and degrading the resulting copolymer with RNase A and spleen phosphodiesterase to give, inter alia, uridine 2′,3′-cyclic phosphorothioate. The latter product was shown to be the endo isomer by high-performance liquid chromatography. No evidence for the presence of the exo isomer was obtained. It can thus be concluded that the *Sp* diastereomer of ADPαS polymerizes with inversion of configuration at phosphorus without racemization to give a phosphorothioate diester bond with the *Rp* configuration.

Recently, we reported on the use of diastereomeric phosphate analogues for the elucidation of the stereochemical course of enzymatic internucleotide bond formation (Eckstein et al., 1976, 1977). It was found that only one of the two diastereomers of adenosine 5′-*O*-(1-thiotriphosphate), the one arbitrarily designated as isomer A, was accepted as a substrate by *Escherichia coli* DNA-dependent RNA polymerase as well as by Baker's yeast tRNA nucleotidyl transferase. Moreover, in both cases, the internucleotide phosphorothioate diester bond was shown by enzymatic analysis to possess the *R* configuration around the phosphorus atom. Since very recently we also know that the A diastereomer of ATPαS has the *S* configuration at the phosphorus atom (Burgers & Eckstein, 1978). This knowledge, together with our previous results, allows us to determine the complete stereochemistry of action of nucleotidyl transferases.

In this paper we report on the polymerization of adenosine 5′-*O*-(1-thiodiphosphate) (ADPαS,[1] Figure 1) catalyzed by polynucleotide phosphorylase from *Micrococcus luteus* and on the stereochemistry of action of this enzyme.

## Materials and Methods

Nucleoside diphosphates were purchased from Waldhof, Mannheim, Germany, and purified over a DEAE-Sephadex column (20 g of DEAE-Sephadex A25/100 mg of product). A linear gradient of 0.05 to 0.4 M triethylammonium bicarbonate (TEAB; 500 mL of each) was applied. [$^{14}$C]UDP and [$^{14}$C]ADP were products from Radiochemical Centre, Amersham, and were used without further purification.

ApU, $Ap_5A$, *M. luteus* polynucleotide phosphorylase (30 units/mg), ribonuclease A, spleen phosphodiesterase (2 units/mg), and alkaline phosphatase (400 units/mg) were obtained from Boehringer, Mannheim, Germany. Rabbit muscle myosin was a gift of Dr. H. Wiedner (Göttingen).

Polygram Cell 300 PEI/UV 254 Fertigfolien from Macherey & Nagel, Düren, Germany, were developed in system A (0.75 M $KH_2PO_4$, pH 3.5) and DC-Fertigplatten Cellulose F from Merck, Darmstadt, Germany, in system B (1 M $NH_4OAc$–EtOH, 3:7, v/v).

The high-performance liquid chromatograph employed was a Packard–Becker 8200 chromatograph, equipped with a Packard 1170 UV detector operating at 254 nm and a Servogor RE 511 recorder. The strong anion exchanger Nucleosil 10 SB from Macherey & Nagel was stirred three times with buffer A (0.5 M $NH_4OAc$, pH 4.4) and decanted to remove the fine particles and then packed into a stainless steel column (40 cm × 2 mm) according to the slurry method. Isocratic elution of the column was normally performed with the same buffer [however, elution of ADPαS (Figure 2) and elution of the products of the nearest-neighbor analysis were effected with buffer B (0.25 M KCl, 0.05 M $KH_2PO_4$, pH 4.5)]. The flow rate was 1.0 mL/min at pressures of 180–220 atm.

*Adenosine 5′-O-(1-Thiodiphosphate). (1) Sp Diastereomer.* Rabbit muscle myosin (5 mg/mL; 0.3 mL) was added to an

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[1] Abbreviations used: ADPαS,*Sp* and ADPαS,*Rp*, diastereomers of adenosine 5′-*O*-(1-thiodiphosphate); ATPαS,*Sp*, *Sp* diastereomer of adenosine 5′-*O*-(1-thiotriphosphate); $Ap_5A$, $P^1$,$P^5$-di(adenosine 5′-)-pentaphosphate; Up(S)A, uridyl(3′-5′)adenyl *O*,*O*-phosphorothioate; U>pS, uridine 2′,3′-cyclic *O*,*O*-phosphorothioate; U>p, uridine 2′,3′-cyclic phosphate.

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