or absence of serum proteins may not explain the nonlinear steroid production we observed during short term incubations.

The incubation of cells with fresh solutions of ACTH or dbcAMP in NSC medium may minimize the exposure of the cells to accumulating metabolic products, including acids, proteins, and steroids. The necessity for this precaution was indicated by the observations that ACTH-stimulated steroidogenesis diminished upon exposure of isolated rat and bovine adrenal cells to physiological concentrations of exogenous corticosterone or cortisol[26]. It seems unlikely that the nonlinear steroid production in our study resulted from an effect of secreted steroids on the response of the cells to stimulation. Because stimulated cells incubated for long periods in SC medium[12,20] may be exposed to increasing concentrations of steroids[26], further studies are required in order to determine an optimum experimental design that maximizes linear steroid production in response to continuous stimulation, but minimizes exposure to accumulating metabolic products.

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Effect of 3,5,3′-tri-iodothyronine on cellular growth and oxygen consumption in neonatal rat brain

Rema R. Rajan and S.S. Katyare

Biochemistry and Food Technology Division, Bhabha Atomic Research Centre, Bombay-400085 (India), 18 September 1981

Summary. The effect of thyroid deficiency and treatment with tri-iodothyronine (T_3) on oxygen consumption by neonatal (6-day-old and 15-day-old) rat brain was examined using glutamate, β-hydroxybutyrate, succinate and ascorbate + TMPD as substrates. The respiration rates decreased significantly in 15-day-old hypothyroid pups. Treatment of normal and of hypothyroid pups with T_3 resulted in a significant increase in the respiration rate at both ages. Respiration rates with glucose as the substrate were not affected under these conditions.

The role of thyroid hormones in regulation of cell formation and maturation during the postnatal development of the brain in rats is well recognized[1-3]. Their role in the regulation of oxygen consumption in this tissue, however, is not clearly understood. Barker[4] reported that while oxygen consumption in tissues such as liver, diaphragm, kidney, heart, salivary gland and pancreas was significantly stimulated by treatment with thyroid hormones, brain, spleen and testis did not respond to this treatment in the adult rat. Reiss et al.[5], in their subsequent studies, were able to show that treatment with thyroid hormones could effectively bring about a stimulation of oxygen consumption in neonatal rat brain. In more recent studies, however, Schwartz and Oppenheimer[6] were unable to confirm these findings and concluded that oxygen consumption in the brain is not responsive to thyroid hormone treatment even in the neonatal rats.

Since the reports on thyroid hormone effect on oxygen consumption in the brain seem to be contradictory, we decided to re-examine this problem by studying the effect of tri-iodothyronine (T_3) on neonatal rats on the 3rd and 11th days after birth. Control and hypothyroid pups were used for these studies and substrates such as glutamate, β-hydroxybutyrate, succinate and ascorbate + TMPD, which are directly metabolized by the electron transport system were used in addition to glucose, which was the only respiratory substrate tested by Reiss et al.[5] and Schwartz and Oppenheimer[6]. These studies have clearly shown that in normal as well as hypothyroid neonatal rats, treatment with T_3 significantly enhances oxygen consumption rates; oxygen consumption with glucose as a substrate, however, was not influenced under these conditions indicating that possibly glucose may not be an adequate substrate for such studies.

Materials and methods. Adult female rats (175 g b.wt) were mated and the morning that spermatozoa were detected in the vaginal tract was considered as day 1 of pregnancy. The control animals had access to food and water ad libitum. In order to produce hypothyroidism, the pregnant females were placed on a diet containing 0.3% propylthiouracil

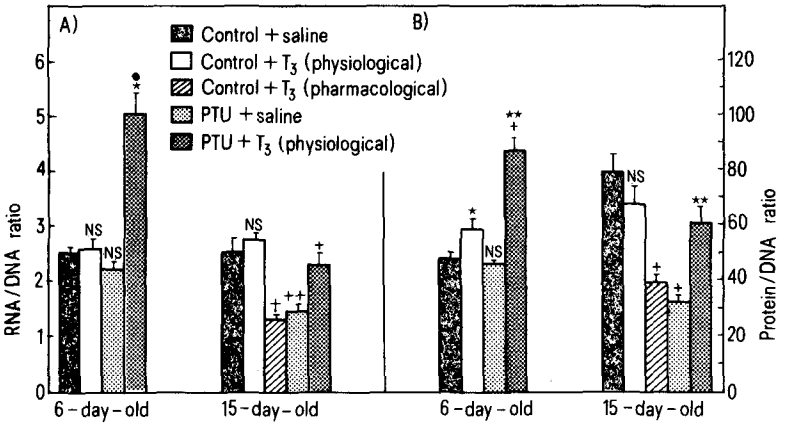

Figure 1. Effect of hypothyroidism and T_3 treatment on the ratios of RNA/DNA and protein/DNA in the brains of neonatal rats. Values are given as mean ± SEM of 11-25 or 5-8 independent observations respectively for 6-day-old and 15-day-old pups. A RNA/DNA ratio; $^{+}p<0.05$ compared with (PTU + saline); $^{++}p<0.02$ compared with (control + saline); $p<0.01$ compared with (control + saline); $^{\dagger}p<0.001$ compared with (control + saline); $\bullet p<0.001$ compared with (PTU + saline); NS, not significant compared with (control + saline). B Protein/DNA ratio: $*p<0.05$ compared with (control + saline); $^{+}p<0.001$ compared with (control + saline); $**p<0.001$ significant compared with (control + saline).

(PTU) and were given 0.001% PTU in their drinking water from the 18th day of gestation onwards[7,8]. Experiments were conducted on 3-day-old and 11-day-old pups which were injected i.p. for 3 and 4 consecutive days respectively either with 25 µl each of saline or T_3 solution (made up in saline by adjusting pH to 8.2 with NaOH) and killed on the 6th and 15th day of life. These pups are hereafter referred to as 6-day-old and 15-day-old pups respectively. Physiological (25 µg/100 g b.wt)[9] and pharmacological (100 µg/100 g b.wt)[10] doses of T_3 were used as indicated in the respective tables and figures, on the basis of average weight. The young animals were kept with the dams till the day of the experiments. At the end of the treatment period, the pups were killed by decapitation and their brains were quickly removed and placed in a beaker containing chilled 0.32 M sucrose, tissues from 2 to 3 pups being pooled when the 6-day-old neonates were used. 20% homogenates were made in 0.32 M sucrose as described previously[11].

Whenever necessary, brain slices were made using a Stadie-Riggs tissue slicer, as described by Schwartz and Oppenheimer, in Krebs-Ringer phosphate buffer, pH 7.4[6]. Oxygen consumption experiments were carried out in a Gilson oxygraph, model KM, using a Clark-type oxygen electrode. Krebs-Ringer phosphate buffer pH 7.4 was used as a respiration medium[6,12]. Tissue homogenate samples containing approximately 5-7 mg protein were incubated in the respiration medium and oxygen consumption rates were recorded using L-glutamate (30 mM), DL-β-hydroxybutyrate (60 mM), succinate (60 mM) and L-ascorbate (5 mM) + TMPD (0.1 mM) as the substrates in a total volume of 1.5 ml at 25 °C, optimum substrate concentrations having been determined in separate experiments. Respiration studies with brain slices were carried out essentially as described by Schwartz and Oppenheimer[6] except that Krebs-Ringer phosphate buffer pH 7.4, both with and without glucose (1 mg/ml) was used.

Extraction of RNA and DNA from brain homogenates was according to Schmidt and Thannhauser[13] and their estimations were carried out by following the published standard procedures[14,15]. Protein was estimated according to Lowry et al.[16] using crystalline bovine serum albumin as a standard. Student's t-test was used to determine the statistical significance of the differences between the means.

Results and discussion. In preliminary studies we ascertained that the desired thyroid state was achieved after feeding PTU or treatment with T_3. This was evaluated in terms of normal parameters of growth such as brain weight and body weight, and the content of macromolecular substances in the brain[1-3,7,8,17]. These results are given in tables 1 and 2.

It can be noted (table 1) that in both 6-day-old and 15-day-old pups, the body as well as brain weights decreased after feeding PTU and they could be partially restored to normal after treatment with physiological doses of T_3. Treatment of the controls with a physiological dose of T_3 by itself had not much effect on the body weight but it resulted in a decrease in the brain weight. Treatment of 15-day-old controls with pharmacological dose of T_3 also produced similar results.

Results for the specific contents of the macromolecules, protein, RNA and DNA in the brains of the 6-day-old and 15-day-old pups (expressed as mg/g tissue) are given in table 2. It can be noted that the altered thyroid state did not significantly affect the cerebral protein content in the 6-day-old pups. The cerebral RNA content was also more-or-less unaffected except in the case of the controls receiving a physiological dose of T_3 where a small decrease in

Table 1. Effect of thyroid status on body weight and brain weight in neonatal rats

Treatment[a]	Age	Body weight g	Brain weight g	% Body weight
Control + saline (37)	6 days	11.24 ± 0.193	0.599 ± 0.013	5.36 ± 0.086
Control + T_3-physiological (23)		11.00 ± 0.179 (NS)	0.524 ± 0.015[d]	4.78 ± 0.154[d]
PTU-fed + saline (28)		6.77 ± 0.096[d]	0.405 ± 0.009[d]	6.00 ± 0.139[d]
PTU-fed + T_3-physiological (17)		8.35 ± 0.183[d,e]	0.513 ± 0.010[d,e]	6.16 ± 0.113[d] (NS)*
Control + saline (8)	15 days	22.02 ± 0.557	1.244 ± 0.018	5.67 ± 0.105
Control + T_3-physiological (12)		19.57 ± 0.310[c]	1.100 ± 0.018[d]	5.68 ± 0.056 (NS)
Control + T_3-pharmacological (12)		20.97 ± 0.420 (NS)	1.113 ± 0.014[d]	5.32 ± 0.092[b]
PTU-fed + saline (7)		10.97 ± 0.314[d]	0.779 ± 0.018[d]	7.12 ± 0.200[d]
PTU-fed + T_3-physiological (8)		13.45 ± 0.297[d,e]	0.950 ± 0.021[d,e]	7.12 ± 0.153[d] (NS)*

The values are given as mean ± SEM. [a] The figures in parentheses indicate the number of animals. [b] $p<0.05$ compared with saline-injected controls; [c] $p<0.002$ compared with saline-injected controls; [d] $p<0.001$ compared with saline-injected controls; [e] $p<0.001$ compared with PTU-fed, saline-injected animals; NS, not significant compared with saline-injected controls; NS*, not significant compared with PTU-fed, saline-injected animals.

total RNA content was evident. PTU feeding did not affect the specific DNA content. Similar observations have also been reported by other investigators[2]. However, treatment of both control and hypothyroid pups with physiological doses of T_3 resulted in a significant decrease in the DNA content. It may, however, be mentioned here that the total content of protein, RNA and DNA, calculated on the basis of total brain weight was significantly lower for the hypothyroid pups as compared to the normals.

In the 15-day-old pups (table 2) PTU feeding resulted in a significant decrease in the protein content in the brain; the RNA content had increased only marginally in these pups and was not affected by subsequent treatment with a physiological dose of T_3. The total RNA content, however, once again, was significantly low in the PTU-fed groups as compared to the normals. In the control group, on the other hand, after treatment with T_3, the RNA content increased significantly; however, in those receiving pharmacological doses of T_3 it increased only marginally. The DNA content increased 2-fold in the hypothyroid pups and was brought back to the normal value after T_3 treatment (physiological dose). In the control group, physiological doses of T_3 had no significant effect on the DNA content in the tissue. Similar findings have also been reported by Patel et al.[3]. Pharmacological doses, however, resulted in a approximately 2-fold increase in the tissue DNA content.

We tried to evaluate the information on macromolecular contents given in table 2 in terms of ratios of RNA/DNA and protein/DNA which are taken as indices of cell size[17]. These data are shown in figure 1. It is apparent that in the 6-day-old pups the treatment with PTU did not significantly affect the cell size assessed either in terms of RNA/DNA or protein/DNA ratios. In this connection it is interesting to note that the level of circulating T_3 in pups at 3–6 day of age is only 25–40% of that found in 15-day-old pups[6]. PTU feeding, therefore, did not significantly affect the cell size. Treatment of controls with a physiological dose of T_3 did not alter the cell size greatly either. However, when the hypothyroid pups were given physiological doses of T_3, there was an approximately 2-fold increase in the cell size as judged in terms of RNA/DNA and protein/DNA ratios. We are unable to explain at the present time this rather large increase in the cell size following T_3 treatment of the hypothyroid pups.

In the 15-day-old pups, on the other hand, PTU feeding resulted in a significant decrease in the cell size (50% decrease) which could be restored almost to the normal after treatment with physiological dose of T_3 (fig. 1). In the control pups, a physiological dose of T_3 did not significantly affect cell size but surprisingly, the pharmacological dose was quite effective in bringing about a significant reduction in the cell size (50% decrease).

The foregoing observations therefore indicate that the desired thyroid state was attained in the pups after various treatments as assessed in terms of cellular parameters of growth and development[1-3,7,8,17].

In the follow-up studies we ascertained the effect of thyroid status on oxygen consumption in the brain using various substrates as outlined in the 'Materials and methods' section. These results are presented in figure 2. It can be noted that respiration rates in general were low in the 6-day-old pups but increased with age. This is in agreement with observations of other researchers[18]. PTU feeding only marginally affected the respiration rates with glutamate, β-hydroxybutyrate and ascorbate+TMPD, the changes, however, not being statistically significant. With succinate the respiration rate decreased by 20%. Treatment of both control as well as PTU-fed pups with physiological doses of T_3 resulted in an overall 2-fold increase in the respiration

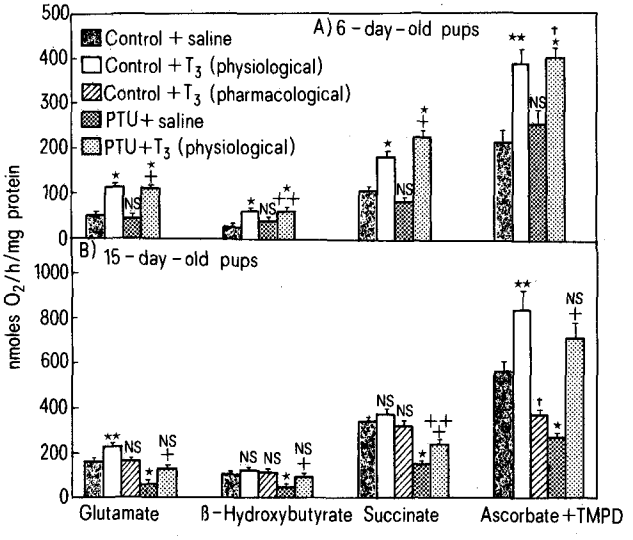

Figure 2. Effect of hypothyroidism and T_3 treatment on respiration rates in neonatal rat brain. Values are given as mean ± SEM of 10–19 or 6–8 independent observations respectively for 6-day-old and 15-day-old pups. *A* $^{++}p<0.01$ compared with (PTU+saline); $^{**}p<0.002$ compared with (control+saline); $^{+}p<0.002$ compared with (PTU+saline); $^{*}p<0.001$ compared with (control+saline); $^{+}p<0.001$ compared with (PTU+saline); NS, not significant compared with (control+saline). *B* $^{**}p<0.05$ compared with (control+saline); $^{+}p<0.01$ compared with (control+saline); $^{++}p<0.002$ compared with (control+saline); $^{*}p<0.001$ compared with (control+saline); $^{+}p<0.001$ compared with (PTU+saline); NS, not significant compared with (control+saline). Note that the scales for A and B are different.

Table 2. Effect of thyroid status on the contents of protein, RNA and DNA in the brains of neonatal rats

Treatment	Age	Protein (mg/g tissue)	RNA	DNA
Control + saline	6 days	63.11 ± 2.13 (15)	3.50 ± 0.14 (24)	1.54 ± 0.11 (25)
Control + T_3-physiological		66.66 ± 2.85 (10) (NS)	2.86 ± 0.28 (17) (NS)	1.17 ± 0.08 (19)[c]
PTU-fed + saline		68.05 ± 1.69 (11) (NS)	3.50 ± 0.13 (12) (NS)	1.59 ± 0.06 (11) (NS)
PTU-fed + T_3-physiological		66.51 ± 1.58 (11) (NS) (NS)*	3.87 ± 0.16 (16) (NS) (NS)*	0.77 ± 0.11 (20)[e,f]
Control + saline	15 days	83.61 ± 4.36 (7)	2.65 ± 0.10 (7)	1.10 ± 0.14 (7)
Control + T_3-physiological		82.28 ± 3.02 (6) (NS)	3.34 ± 0.06 (6)[e]	1.23 ± 0.06 (6) (NS)
Control + T_3-pharmacological		91.30 ± 1.04 (6) (NS)	2.89 ± 0.13 (6) (NS)	2.34 ± 0.16 (6)[e]
PTU-fed + saline		66.08 ± 1.89 (6)[c]	2.94 ± 0.05 (6)[a]	2.19 ± 0.27 (6)[c]
PTU-fed + T_3-physiological		75.12 ± 1.52 (8) (NS)[d]	2.83 ± 0.05 (7) (NS) (NS)*	1.30 ± 0.12 (7) (NS)[b]

The values are given as mean ± SEM of the number of observations indicated in parentheses. [a] $p<0.05$ compared with saline-injected controls; [b] $p<0.02$ compared with PTU-fed, saline-injected animals; [c] $p<0.01$ compared with saline-injected controls; [d] $p<0.01$ compared with PTU-fed, saline-injected animals; [e] $p<0.001$ compared with saline-injected controls; [f] $p<0.001$ compared with PTU-fed, saline-injected animals; NS, not significant compared with saline injected controls; NS*, not significant compared with PTU-fed, saline-injected animals.

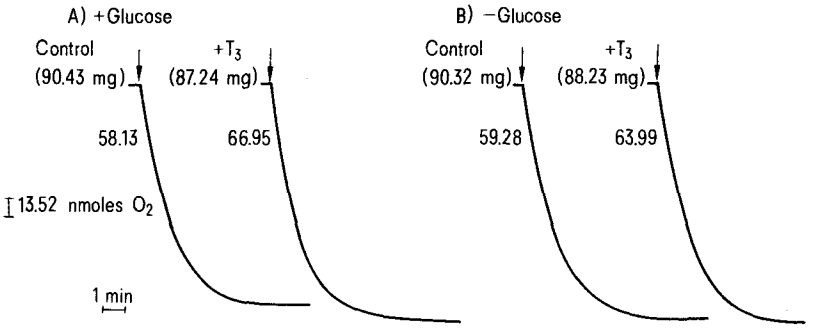

Figure 3. Typical oxygraph traces showing respiration rates in brain slices of: *A* Control, and *B* T_3-treated 15-day-old rat pups. Experimental details are as described in the text. Figures on the left hand side of the traces represent the corresponding respiration rates for the initial linear portions of the traces and are given as nmoles O_2/h/mg wet wt. Arrows indicate the point where the tissue slices were added to the respiration medium.

rates with all the substrates examined. This may once again relate to the low levels of circulating T_3 in the serum at the 6th day of age compared to those on the 15th day as cited above[6]. I.p. injected T_3 was, therefore, equally effective in both control and hypothyroid pups in stimulating the respiration rates.

In the case of 15-day-old pups, thyroid deficiency resulted in lowering the oxygen consumption by about 55–60%, and treatment with T_3 was effective in restoring the respiratory rates to about 70–85% of the values for the normal controls for glutamate, β-hydroxybutyrate and succinate; with ascorbate + TMPD, the rate was in fact higher than in controls (45% increase). Treatment of control pups with physiological doses of T_3 brought about a marginal stimulation of oxidation rates with glutamate and ascorbate + TMPD without having any effect on the oxidation rate of β-hydroxybutyrate and succinate. The pharmacological dose, however, had no appreciable effect on respiration rates except in the case of ascorbate + TMPD where a 35% decrease was evident. Other studies from our laboratory have similarly shown that higher pharmacological doses of T_3 *in fact result in decreased oxygen consumption rates in* adult rat brain[19].

We next tried to repeat some of the experiments of Schwartz and Oppenheimer[6]. For these studies we essentially followed the protocol given by Schwartz and Oppenheimer[6]; 11-day-old pups were treated with pharmacological doses of T_3 for 4 days and killed on the 15th day. Oxygen uptake by the brain slices was determined in the presence and absence of glucose (1 mg/ml) in the respiration medium. These results are given in table 3. It can be noted that as found by Schwartz and Oppenheimer[6], the oxygen consumption rates in glucose medium for the control and T_3-treated groups were practically identical. Surprisingly, however, the rates appeared to be virtually unaffected even when glucose was omitted from the respiration medium. This may probably indicate that endogenous substrate(s) and not glucose was/were being utilized for cellular respiration. Such an assumption is further substantiated by the fact that when the slices were pre-incubated for 60 min prior to a study of oxygen consumption, the respiration rates decreased by about 25–30% (table 3) possibly because the endogenous substrates leaked out during the pre-incubation period. This is further exemplified in oxygraph traces shown in figure 3 where it is apparent that initial respiration rates in brain slices from normal and T_3-treated pups were practically identical, irrespective of the presence or absence of glucose. The rates, however, declined with time and came practically to a standstill as the endogenous substrates were depleted. Linear respiration rates in fact could be obtained by adding glutamate or succinate to the slices after respiration had ceased (results not shown). From the foregoing studies it would therefore seem that for such respiration studies one has to be cautious while using glucose as the sole respiratory substrate; it may be advisable instead to use other substrates which are more directly metabolized by the electron transport system as shown in figure 2. In this connection it is also noteworthy that β-hydroxybuturate is an excellent alternative fuel for neonatal rat brain[20,21].

Table 3. Effect of T_3 on oxygen consumption in brain slices of 15-day-old rat pups with glucose as substrate

Time (min)	Animals[a]	Oxidation rate (nmoles O_2/h/mg wet wt)	
		+ glucose	− glucose
0	Control (4)	43.51 ± 4.94	41.90 ± 4.58 (NS)
	T_3-injected (5)	40.75 ± 4.00 (NS)	39.96 ± 3.76 (NS)
60	Control (4)	32.00 ± 1.38	26.80 ± 3.01[b]
	T_3-injected (5)	28.04 ± 0.54[c]	31.07 ± 3.27 (NS)

The experimental details are described under 'Materials and methods'. Approximately 75–85 mg of tissue slices were used per experiment. Linear respiration rates were recorded for 7–10 min. The values are given as mean ± SEM. [a] Figures in parentheses represent the number of animals; [b] $p < 0.05$ compared to control at zero h; [c] $p < 0.02$ compared to T_3-injected at zero h; NS, not significant.

Although glucose is considered as a primary source of energy for the brain, it must be recognized that glucose has to be metabolized through the hexose monophosphate shunt and/or the glycolytic and tricarboxylic acid cycles. Other studies from our laboratory have indeed shown that to get good respiration rates with glucose, a pre-incubation for about 20 min is necessary before any oxygen consumption could be obtained (R.R. Rajan and S.S. Katyare, unpublished work).

In conclusion, the results of the present studies have clearly shown that even in the neonatal rat brain the rates of oxygen consumption are greatly influenced both by the thyroid deficiency state of neonates and their treatment with thyroid hormone and that one has to exercise caution in using glucose as the sole respiratory substrate for such studies.

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Insulin stimulation of glucose and amino acid transport in mouse fibroblasts with elevated membrane microviscosity[1]

I. Yuli, S. Incerpi[2], P. Luly[2] and M. Shinitzky[3]

Department of Membrane Research, The Weizmann Institute of Science, Rehovot (Israel), 12 January 1982

Summary. Basal and insulin-stimulated transport of 2-deoxy glucose and of α-aminoisobutyric acid in mouse 3T3 fibroblasts were modulated by increasing the lipid microviscosity of the cell plasma membrane. The kinetics indicate that the insulin effect is induced either by recruitment of new transport carriers or by reduction of the translocation activation energy.

Carrier-mediated glucose transport in human erythrocytes and mouse fibroblasts of modified membrane microviscosity, $\bar{\eta}$, does not obey simple diffusion considerations[4]. Upon slight increase in $\bar{\eta}$ the maximal transport rate, V_{max}, reaches a peak activity beyond which further increase in $\bar{\eta}$ reduces it. The effect of increased $\bar{\eta}$ could be accounted for by 2 counteracting trends; a reduced turnover rate of each transport carrier and an apparent increased number of operating carrier sites[4]. In the following study we examined the insulin stimulation of transport of 2-deoxy glucose (2dG) and of α-aminoisobutyric acid (AIB) in 3T3 mouse fibroblasts[5,6] with increased membrane microviscosity[4]. Transport experiments were carried out at 37 °C with quiescent cell-monolayers in which the plasma membrane microviscosity was selectively increased by incorporation of cholesteryl hemisuccinate (CHS) under mild conditions[4]. Steady-state fluorescence polarization with DPH as a probe, in addition to a special quenching technique, were employed to determine and resolve the $\bar{\eta}$ value at 37 °C of the cell plasma membranes in the monolayers[4]. Although $\bar{\eta}$ is a complex combination of dynamics and order of the membrane hydrocarbon region[7] it is of a direct relevance to membranal activities[8]. A full description of the experimental details and rationale is given in a previous publication[4]. At each level of membrane viscosity transport experiments were repeated 6–12 times. Figure 1 summarizes the effect of increased membrane microviscosity on the maximal rate of 2dG transport (V_{max} at 37 °C) in the absence and in the presence of 100 ng/ml of insulin[9]. As shown, within the experimental microviscosity range, the effect of insulin on the maximal transport rate of 2dG is increased by about 50%. The patterns described in figure 1 reveal a distinct maximal V_{max} value at about 30–50% increase in the apparent $\bar{\eta}$-value. Analogous experimental results for AIB transport are shown in figure 2.

Following our previous approach[4] for analysing the effect of $\bar{\eta}$ on a membranal activity, V_{max} can be presented as the product of the transport rate constant, k_{tr}, and the actual concentration of the operating carriers, C_+:

$$V_{max} = k_{tr} \cdot C_+ = k_{tr} \cdot \alpha \cdot C_0 \qquad [1]$$

where α is the accessible fraction of the total operateable carriers C_0, which is operationally dependent on $\bar{\eta}$.

Analysis of the dependence of α and k_{tr} on $\bar{\eta}$ leads to the following general expression[4]:

$$\frac{1}{V_{max}} = A \cdot \eta \cdot \frac{1+\tilde{\eta}^{-m}}{1+f \cdot \tilde{\eta}^{-m}} \qquad [2]$$

where $\tilde{\eta}$ is the normalized lipid microviscosity, $\bar{\eta}$, in units of $\bar{\eta}_{1/2}$, the latter corresponds to half accessibility ($\tilde{\eta} = \bar{\eta}/\bar{\eta}_{1/2}$), and m is a plasticity parameter which characterizes the degree of cooperativity between the lipid viscosity and the statistical accessibility of the carrier sites.

Fitted parameters (± SE) for the dependence of the maximal rate of transport (V_{max} at 37 °C) on membrane microviscosity ($\bar{\eta}$), according to Eq. 2

Ligand	Insulin stimulation (100 ng/ml)	A $\left(\frac{\text{nmole} \cdot \text{poise}}{10^6 \text{ cell} \cdot \text{min}}\right)^{-1}$	$\bar{\eta}_{1/2}$, 37 °C (poise)	m	f
2dG	–	3.2 (±0.2)	1.41 (±0.03)	4.8 (±1.1)	~0
2dG	+	2.6 (±0.2)	1.42 (±0.03)	4.7 (±1.1)	~0
AIB	–	0.68 (±0.02)	1.32 (±0.03)	>8	~0
AIB	+	0.24 (±0.01)	1.32 (±0.02)	>8	~0