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SUGAR UPTAKE BY CYTOCHALASIN B IN
CULTURED CHICK FIBROBLASTS

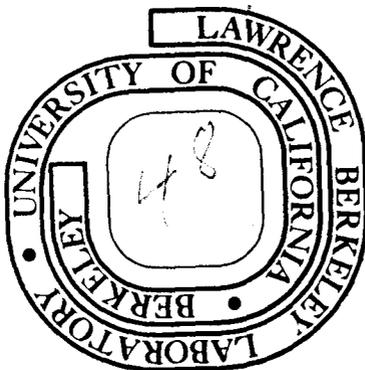
David Dolberg, Mina J. Bissell and James A. Bassham

October 31, 1974

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SELECTIVE INHIBITION OF FACILITATED MODE OF SUGAR UPTAKE BY CYTOCHALASIN B
IN CULTURED CHICK FIBROBLASTS

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Running Title: Cytochalasin B and the Facilitated Sugar Uptake

5 tables

6 figures

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SUMMARY

Cytochalasin B, an inhibitor of sugar transport in cultured cells, inhibits only the facilitated mode of 2-deoxyglucose transport in chicken embryo fibroblasts. The drug thus may be used to examine separately the passive and the facilitated modes of sugar uptake. Factors such as growth rate, glucose concentration, cell density, and viral transformation were shown to affect not only the overall rate of hexose transport, but also the rate of one mode relative to the other.

INTRODUCTION

The ability of cytochalasin B (CB), a mold metabolite [1], to inhibit hexose transport in cultures of chick embryo fibroblasts (CEF) [2] and other cell types [3-5] is well documented. The nature of this inhibition, however, is currently a matter of dispute [3,5,6]. A clearer understanding of the underlying mechanism of inhibition would greatly enhance the usefulness of CB in the study of hexose transport.

It has been reported that glucose is transported by two separate modes in CEF [7] and Novikoff Rat Hepatoma Cells [8], a high affinity facilitated mode and a low affinity passive mode. The facilitated transport by definition requires a carrier protein and is thus saturable. In passive diffusion, the solute moves across a concentration gradient, does not require a carrier protein, and is not saturable. Evidence cited here confirms that these two modes of hexose transport exist in CEF. We further show that CB selectively inhibits facilitated diffusion.

Using CB to distinguish between these two modes of transport, we examined the uptake kinetics of 2-deoxy-glucose (2dg) in both normal and Rous sarcoma virus transformed CEF. It was found that growth rate, cell density, and other factors in the cell environment may alter not only the overall transport rate, but also the rate of transport of one mode relative to the other. Therefore, while a difference in the degree of inhibition of hexose transport by CB may be demonstrable between normal and transformed cells [9], this difference can be made to disappear if the environmental conditions are changed to favor one or the other mode of transport.

METHODS

Primary cultures, free of resistance-inducing factor, were prepared from 10 day old C/O or C/B type SPF chick embryos [10], as previously described [11]. In brief, embryos were decapitated, minced, washed with tris-saline buffer, and digested with 0.25% trypsin. After 15 min the suspended cells were poured into a "stop bath" containing 2/3 cold medium 199 (Gibco) and 1/3 calf serum (Microbiological Associates, Inc.). This process was repeated twice. The single cells were then divided into two groups and plated in 100 mm culture dishes at 8×10^6 cells per plate in medium 199. Two percent tryptose phosphate broth (Gibco), 1% calf serum, and 1% chicken serum (Microbiological Associates, Inc.), abbreviated as (2-1-1), were added to the medium. One group was infected 4 h after primary seeding with 0.2 ml of Schmidt-Ruppin strain of Rous sarcoma virus (5×10^6 focus forming units/ml). Both groups were then treated equally. No fungicides were added to the medium, as it has been demonstrated that fungicides alter transport characteristics in CEF in culture [12].

Secondary cultures were plated in 35 mm plates at various cell densities and different growth conditions (2-1-1), (10-4-1; using the same convention as above) for serum stimulated growth [13,14], or (2-0-0) for serum depleted growth, in order to obtain the desired combination of cell density and growth rate. An additional 1% glucose was added to the fast growing and transformed cells to prevent glucose depletion of the medium [15]. Two days after secondary platings, the cultures were used for experiments. Growth rates were monitored by giving a one-hour pulse of ^3H -thymidine (Tdr; $2 \mu\text{C}/\text{ml}$ medium) and by measuring its rate of incorporation into DNA [16], taking into account any changes in the pool size, and by monitoring cell number with a Coulter counter.

The rate of uptake of [^3H]-2-deoxy-glucose (2dg), a non-metabolizable glucose analog, was used as a measure of hexose uptake [13]. Plates were washed three times in warm, glucose-free Hank's buffer. [^3H]-2dg was administered in glucose-free medium 199 for the lengths of time noted in the results. The labeled 2dg was then removed and cells were washed three times with cold Hank's buffer prepared with 5.5 mM glucose. The measurement of the uptake of [^3H]-mannitol was performed in the same manner. All radioactive materials were obtained from New England Nuclear Corp.

Lowry solution [17] was added directly to the plates. An aliquot sample was taken for the measurement of radioactivity, and counted in a Packard Model 3375 scintillation counter after addition of 15 ml of Aquasol (New England Nuclear Corp.). Cell protein was determined from another aliquot sample by the method of Lowry *et al.* [17]. Cytochalasin B was obtained from Aldridge Chemical Company, Inc.

RESULTS

The uptake of 2dg was shown to be linear for at least 15 min in the presence or absence of CB (1 $\mu\text{g}/\text{ml}$), and the inhibition was virtually complete 2 min after CB addition. This agrees with data from other laboratories [2]. Addition of CB in concentrations of 1 $\mu\text{g}/\text{ml}$ resulted in 85-90% inhibition of 2dg transport in normal CEF, and greater concentration increased the degree of inhibition only slightly (Fig. 1). Similar results were found for transformed cells. We therefore chose an incubation time of 5 min for measurements of 2dg uptake and a concentration of 1 $\mu\text{g}/\text{ml}$ of CB as the standard parameters for subsequent experiments (unless otherwise specified).

Previous studies have shown that hexoses are transported by at least two separate transport modes in CEF [7]: a high affinity, facilitated mode which is dominant at sub-physiological substrate concentrations (below 1 mM), and a lower affinity passive mode which becomes significant at concentrations approaching those found in vivo. In order to obtain a detailed study of the kinetics of 2dg transport, we chose to look at both concentration ranges of the substrate: a lower range of 10^{-7} to 10^{-5} M (Fig. 3A,B) and a higher range of 10^{-4} to 10^{-2} M (Figs. 2 and 3C,D). Neither normal nor transformed cells show any significant change in K_m when treated with CB (Fig. 3). However, CB does lower V_{max} , especially at higher 2dg concentrations. These changes in the kinetic parameters of transport are usually indicative of non-competitive inhibition. Other data presented later seems to support this conclusion (however, see Discussion).

As the concentration of 2dg was increased, the slope of the velocity curve in untreated cultures approached the slope of the velocity curve in cultures treated with CB (Fig. 2). This appeared to be true in both normal and transformed cells. Further, in cells treated with CB, the relation between 2dg concentration and transport velocity was linear (Fig. 2). This indicated that CB had mainly inhibited the facilitated transport and had not affected the passive mode of uptake. An experiment carried out at very high concentrations of 2dg (concentrations at which passive diffusion would be most dominant), showed no difference between the slope of velocity curves in the presence or absence of CB (Fig. 4). This is consistent with the conclusion that CB does not inhibit passive diffusion.

To test this conclusion further, we examined the effect of CB on the transport of mannitol, a hexitol transported only by passive diffusion [18].

Mannitol has been used previously as a measure of the non-saturable mode of hexose transport in chick cells [7]. In Fig. 5 we see that CB had no effect on the transport of mannitol in either normal or transformed cells, up to the highest concentrations examined (10 mM). This is additional support that CB preferentially inhibits the facilitated mode of transport in CEF.

The velocity obtained in the presence of CB was thus used as a means of correcting our original uptake data (Fig. 2) by subtracting for non-saturable uptake. This enabled us to analyze separately the carrier mediated mode of transport. The result of this manipulation is shown in Fig. 6a and b, and the K_m and V_{max} values calculated from this plot are shown in Table 1, where they are compared with the uncorrected values obtained from Fig. 3.

Since 2dg is transported by both a facilitated and a passive mode, but CB inhibits only the facilitated mode, it follows that the degree of inhibition of 2dg transport by CB depends only on how much of total transport is indeed mediated by facilitated diffusion. It is necessary, therefore, to examine the environmental conditions of the cell for changes which affect not only the total hexose transport, but which may also shift the burden of transport from one mode to the other. The factors considered were glucose deprivation, growth stimulation, and cell density.

Cells incubated in various concentrations of glucose for 8 h prior to the time of assay showed differing sensitivities to CB (Table 2). Starvation increased the total inhibition of 2dg by CB. Increase in overall glucose transport after starvation has been previously demonstrated for chick cells in culture [19]. Note, however, that the velocity in the

presence of CB (i.e., the passive diffusion) has changed very little after starvation. This indicates that the change in overall uptake, caused by starvation, can be accounted for by a change in the facilitated mode of transport.

A change in growth rate also altered the sensitivity of chick cells to CB. Two groups of normal cells (A and B) were prepared in such a way that their cell densities were comparable, but their growth rates, as evidenced by thymidine incorporation (and subsequent cell number), were different. Thymidine incorporation (in the absence of a change in the pool size of thymidine) has been shown to be an accurate index of growth rate in these cells [16]. The faster growing cells showed a higher sensitivity to CB at low concentrations of 2dg (Table 3). We observed little difference in the uptake velocity of the passive mode (transport remaining in the presence of CB) in either group at the lower substrate concentration--thus most of the difference is attributable to a change in facilitated diffusion. As the concentration of 2dg is increased to a range in which passive diffusion becomes more significant, we found stimulation of uptake velocity in both modes in Group A relative to B. This suggests a change in the passive as well as the facilitated mode.

An increase in population density without a comparable change in growth rate has a different effect (Table 3, A and C). In this case, inhibition is similar at low concentrations of 2dg, but at higher concentrations the more populated cultures show a greater sensitivity to CB. This indicates that the two cultures were demonstrating similar activity in the facilitated mode, but the sparser cultures demonstrated more activity in the passive mode.

Examination of cells treated with Rous sarcoma virus shows that both passive and facilitated diffusion increase after transformation, with the facilitated mode playing a more significant role in hexose transport (Table 4). Table 5 summarizes the effects of environmental perturbations on the two modes of hexose transport.

DISCUSSION

Evidence presented here indicates that CB selectively inhibits the facilitated mode of hexose transport in CEF in culture. Conditions which favor or increase the facilitated transport of 2dg relative to its passive diffusion through the membrane will increase the percent inhibition of overall 2dg transport by CB. This is seen especially at lower concentrations of 2dg (below 10^{-4} M), which tend to favor facilitated diffusion. On the other hand, conditions which favor passive diffusion result in decreasing sensitivity to CB as the concentration of substrate in the medium is increased to a range in which passive diffusion becomes more significant. This is true even if the absolute velocity of the facilitated mode is increased. For example, in Table 4 we can calculate the rate of transport of the facilitated mode by subtracting the rate of transport in the presence of CB from the rate of transport in its absence. A comparison of normal and transformed cells incubated with 5 mM 2dg shows a facilitated uptake velocity which is almost twice as high in transformed cells as it is in normal cells. Yet, the passive diffusion in normal cells, relative to the facilitated diffusion, is sufficiently low so that the normal cells still show the greatest percent inhibition after the addition of CB.

The selectivity of CB's effect on the facilitated mode of sugar transport provides us with a method by which we can divide the bimodal process of hexose transport into its component parts. It also suggests that the cause of this inhibition is an interaction between CB and the facilitated transport carrier protein, rather than a general effect on membrane functions. The rate of change of uptake velocity remains the same in the presence and absence of CB up to substrate concentrations of 80 mM (Fig. 5). This is true in both normal and transformed cells. If the nature of the inhibition were competitive, increasing concentrations of substrate would eventually eliminate the observable inhibition, and this does not seem to be the case (at least up to 80 mM). Since the level of CB used is slightly less than saturable levels, this finding tends to indicate that CB is a non-competitive inhibitor of hexose transport as found in other systems [3]. This point, however, needs further verification, as it contradicts findings of other laboratories [2]. The contradictions should also be reexamined in the light of the complexity of the glucose transport system and the nature of the glucose analog used in various experiments (see below).

CB has been shown to be a depolymerizer of microfilaments, a fact which has been used to explain many of the other observed effects of CB [20]. In addition, microfilaments have been shown to be associated with membrane structure and function [21], especially that of microvilli [22]. Recent scanning electron microscopic investigations have shown a correlation between the appearance and disappearance of microvilli and perturbations which are known to affect hexose transport, such as transformation [23] and treatment with insulin [24]. Since the effect of CB on glucose

transport occurs at much lower concentrations than that needed for a visual effect on microfilaments, the two processes may indeed be unrelated.

Recent studies indicate that there are two classes of binding sites for CB in mammalian cells [25]--a high affinity site which may be the hexose transport site and a low affinity site which may be the site affecting cytokinesis and the general morphology of the cells [25]. On the other hand, it cannot be ruled out that microfilaments and/or microvilli may be involved in the maintenance of the active conformation of the hexose transport carrier protein. Again, the complexity of the transport mechanisms in various cell systems makes it difficult to draw conclusions from one cell type to another.

Recent studies have demonstrated the importance of an increasing number of factors in the cell environment on the behavior and metabolism of cells in culture. These factors include the effects of growth and cell densities on the glucose metabolism of CEF [26], the effect of starvation [19], and the effects of glucose concentration in the medium on the transport and metabolism of glucose [15]. The effects of transformation on transport and metabolism of glucose has been the subject of much recent investigation [27]. Evidence presented here shows that changes in these environmental factors can affect the passive and facilitated modes of transport independently to bring about a net change in hexose transport. Alterations in the rate of facilitated diffusion are most likely due to either an increase in the number of transport sites or an increase in the turnover rate of the existing sites [28]. This explanation can account for the increase in facilitated transport in transformed as well as normal cells since the kinetics of the facilitated mode per se shows no significant change in K_m after transformation [7,29,30, and our results]. Alterations in the rate of passive diffusion can be accounted for either by a change in

the concentration of substrate in the medium, or by a change in the membrane surface area available to the medium. The latter may explain why a culture of sparsely populated cells shows a higher rate of passive diffusion (Table 3). Hexose deprivation, however, stimulates transport primarily in the facilitated mode (Table 2). This would agree with the conclusion of Martineau et al. that such a stimulation can be prevented by the use of protein inhibitors [19].

Variable affinities of different hexoses would also alter the apparent inhibition of hexose transport by CB. For example, sugars with lower affinities for transport would tend to emphasize transport by the passive mode. This might explain discrepancies found in other laboratories between the effects of CB on the transport of 2dg and glucose [6], or 2dg and 3-0-methyl glucose [9]. Differences between the effects of CB on normal and transformed cells can be exaggerated or eliminated by changing environmental conditions which favor one or the other mode of transport (Tables 3 and 4), and could possibly account for the differences noted in previous reports [9].

ACKNOWLEDGMENTS

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Table 1. Kinetic parameters of 2dg transport in normal and transformed chick cells in culture

2dg Concentration range	Normal cells		Transformed cells	
	K_m	V_{max}	K_m	V_{max}
A 10^{-7} M- 10^{-5} M	.2	0.1	.2	1.0
B 10^{-4} M- 10^{-2} M	6.3	27.0	5.0	80.0
C Corrected for non-saturable uptake with 1 μ g/ml CB	2.7	8.3	3.3	18.5*

K_m is expressed as 10^{-3} M/liter. V_{max} is expressed as nmoles of 2dg/mg protein/min. Line A was calculated from Fig. 3a and b, Line B from Fig. 3c and d, and Line C from the data on Fig. 6b.

*The magnitude of V_{max} in transformed cells depends on the degree of transformation. In these experiments the cells were 70 - 80% transformed. 100% transformed cells have a tendency to come off the plates in a sheet.

Table 2. Effects of glucose deprivation on the two modes of hexose transport in CEF

8 hr preincubation with:	2dg uptake (dpm/ μ g protein)		% inhibition by CB
	Control	+CB (.25 μ g/ml)	
Medium 199 + no glucose	20.5 \pm 0.9	4.04 \pm 0.2	80.3
" " + 0.55 mM glucose	14.2 \pm 0.7	4.18 \pm 0.7	70.5
" " + 5.5 mM glucose	7.22 \pm 0.03	3.10 \pm 0.1	57.0

Cells were plated at 10^6 cells/35 mm plate, in (2-2-1), 48 hr prior to the experiment. Cells were incubated with the concentration of glucose indicated above, for 8 hr prior to the assay, and the appropriate plates were treated with 0.25 μ g/ml CB during the measurement of [3 H]-2dg uptake, as described in Methods. Average of duplicate plates of two experiments.

Table 3. Effects of growth rate and cell density on the two modes of hexose transport in CEF

	<u>A</u>			<u>B</u>			<u>C</u>		
Cell count	1.4x10 ⁶ *			1.5x10 ⁶ *			3.0x10 ⁶ *		
Incorporation of ³ H-Tdr	52000**			18500**			44000**		
	Velocity of		% inhib.	Velocity of		% inhib.	Velocity of		% inhib.
	2dg uptake***		by CB	2dg uptake***		by CB	2dg uptake***		by CB
Concentrations of 2 dg:	<u>Control</u>	<u>+CB 1 µg/ml</u>		<u>Control</u>	<u>+CB 1 µg/ml</u>		<u>Control</u>	<u>+CB 1 µg/ml</u>	
6x10 ⁻⁷ M	.021 [±] .002	.0038 [±] 0.0005	81.9	.0054 [±] 0.001	.0040 [±] 0.0006	26.0	.020 [±] .001	.003 [±] 0.0005	85.0
5x10 ⁻³ M	84.3 [±] 1.8	34.6 [±] 1.1	58.0	33.4 [±] 0.6	20.6 [±] 0.4	38.3	71.5 [±] 1.2	14.4 [±] 0.8	79.9
10 ⁻² M	131.0 [±] 3.0	77.2 [±] 1.4	41.0	71.6 [±] 1.0	30.0 [±] 0.5	58.0	96.7 [±] 2.1	33.4 [±] 1.0	65.5

A and B were at comparable cell densities and A and C were growing at comparable rates at the time of assay. Secondary cells were plated as follows: A - 5x10⁵ cells/dish in 10-4-1; B - 10⁶ cells/dish in 2-2-1 and the medium was changed to 2-0-0 12 hr before assay; C - 10⁶ cells/dish in 2-2-1.

*Cell count is expressed as number of cells/35 mm dish.

**³H-Tdr incorporation is expressed as dpm/mg protein.

***Velocity is expressed as nmoles of 2dg/mg protein/min.

Average of duplicate plates of two experiments.

Table 4. Effect of transformation on the two modes of hexose transport in cultures of comparable growth rate and density

Concentration of 2dg	Normal			Transformed		
	Control	Velocity +CB (1 $\mu\text{g/ml}$)	% inhibition	Control	Velocity +CB (1 $\mu\text{g/ml}$)	% Inhibition
6×10^{-7} M	$.013^{\pm}.003$	$.002^{\pm}.001$	86.4	$.035^{\pm}.001$	$.0054^{\pm}.0008$	84.4
5×10^{-3} M	$46.2^{\pm}1.0$	$9.9^{\pm}1.9$	78.4	$70.2^{\pm}7.1$	$16.1^{\pm}0.2$	77.9
10^{-2} M	$63.0^{\pm}2.5$	$37.0^{\pm}1.1$	41.4	$145.1^{\pm}14.3$	$39.0^{\pm}1.1$	69.4

Cells were plated as follows: Normal - 10^6 cells/plate in 10-4-1; transformed - plated at 7.5×10^5 cells/plate in 10-4-1. Velocity is expressed as nmoles of 2dg/mg protein/min. Cell count, at the time of the experiment, was 3.1×10^6 cells/plate for normal cells and 4.2×10^6 cells/plate for transformed cells. Growth rate as measured by $^3\text{H-Tdr}$ incorporation into DNA was similar in both cultures - 8200 dpm/mg protein for normal cells; 9700 dpm/mg protein for transformed cells. Average of duplicate plates of two experiments.

Table 5. Summary of the effects of environmental changes on the two modes of hexose transport in chick cells

	Effect on 2dg transport	
	facilitated	passive
Fast growth	++	+
high density	0	-
transformation	+++	+
glucose deprivation	+*	0

*The magnitude of this increase depends on the severity of starvation.

(+) indicates stimulation; (-) indicates retardation; (0) indicates little or no effect.

FIGURE CAPTIONS

Fig. 1. Ordinate: % inhibition of 2dg transport; abscissa: $\mu\text{g/ml}$ of CB. Effect of the concentration of CB on the transport of 2dg. Cells were plated at 10^6 cells/35 mm plate in 2-2-1 (see Methods), and 2dg uptake was measured as described. Average of duplicate plates of 3 experiments.

Fig. 2. Ordinate: velocity of transport of 2dg (nmoles of 2dg/mg protein/min); abscissa: concentration of 2dg in the medium (10^{-3} M).

Transport velocity of 2dg at various substrate concentrations. Cells were plated at 10^6 cells/35 mm plate in 10-4-1. A - normal cells; (\square) presence of CB; (o) absence of CB. B - transformed cells; (\blacksquare) presence of CB; (\bullet) absence of CB.

Fig. 3. Ordinate: $1/V$ (nmoles of 2dg/mg protein/min); abscissa: $1/[S]$ (A&B= 10^{-5} M) (C&D= 10^{-2} M). Lineweaver Burke plots of the uptake kinetics in the presence (\blacksquare) and absence (\bullet) of CB. A&C are normal and B&D are transformed. Normal and transformed cells were plated at 10^6 cells/plate in 10-4-1.

Fig. 4. Ordinate: velocity of 2dg uptake (nmoles of 2dg/mg protein/min); abscissa: substrate concentration (10^{-3} M).

Transport velocity of 2dg in the presence (squares) and absence (circles) of CB at high substrate concentrations. Cells were plated at 10^6 cells/35 mm plate in 10-4-1. a) Normal cells (open symbols); b) transformed cells (closed symbols).

Fig. 5. Ordinate: velocity of mannitol uptake (nmoles of 2dg/mg protein/min);
abscissa: substrate concentration (10^{-3} M).

Transport velocity of mannitol in the presence (squares) and absence (circles) of CB. Cells were plated at 10^6 cells/35 mm plate in 10-4-1. a) Normal cells (open symbols); b) transformed cells (closed symbols).

Fig. 6. a) Ordinate: velocity of 2dg uptake (nmoles of 2dg/mg protein/min);
abscissa: substrate concentration (10^{-3} M).

Transport velocity of the facilitated mode of hexose transport in normal (o) and transformed (●) cells. Obtained by subtracting the transport in the presence of CB from the transport in the absence of CB.

b) Ordinate: $1/V$; abscissa: $1/s$.

Lineweaver Burke plot of the data in 6a.

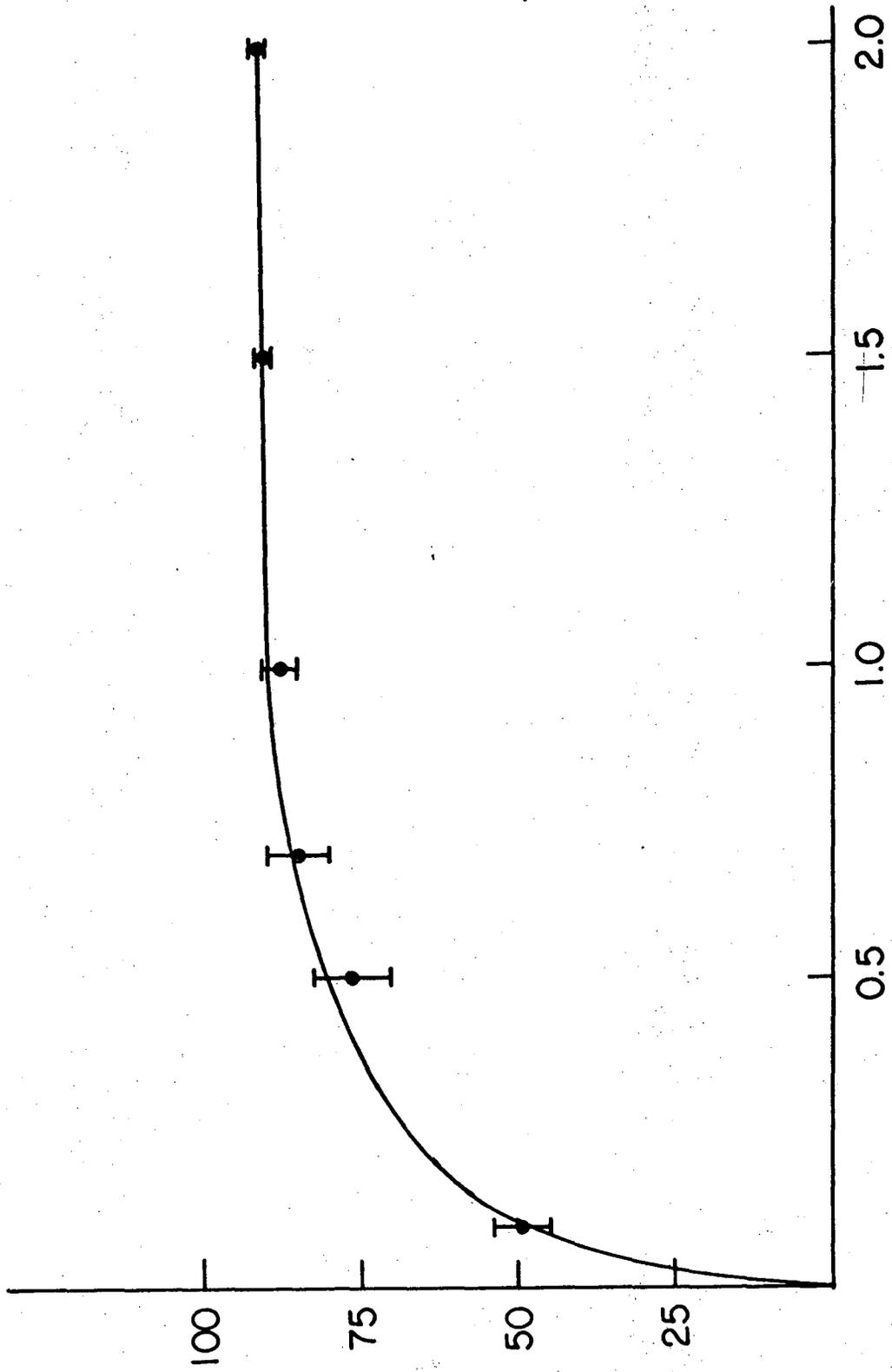


Fig. 1.

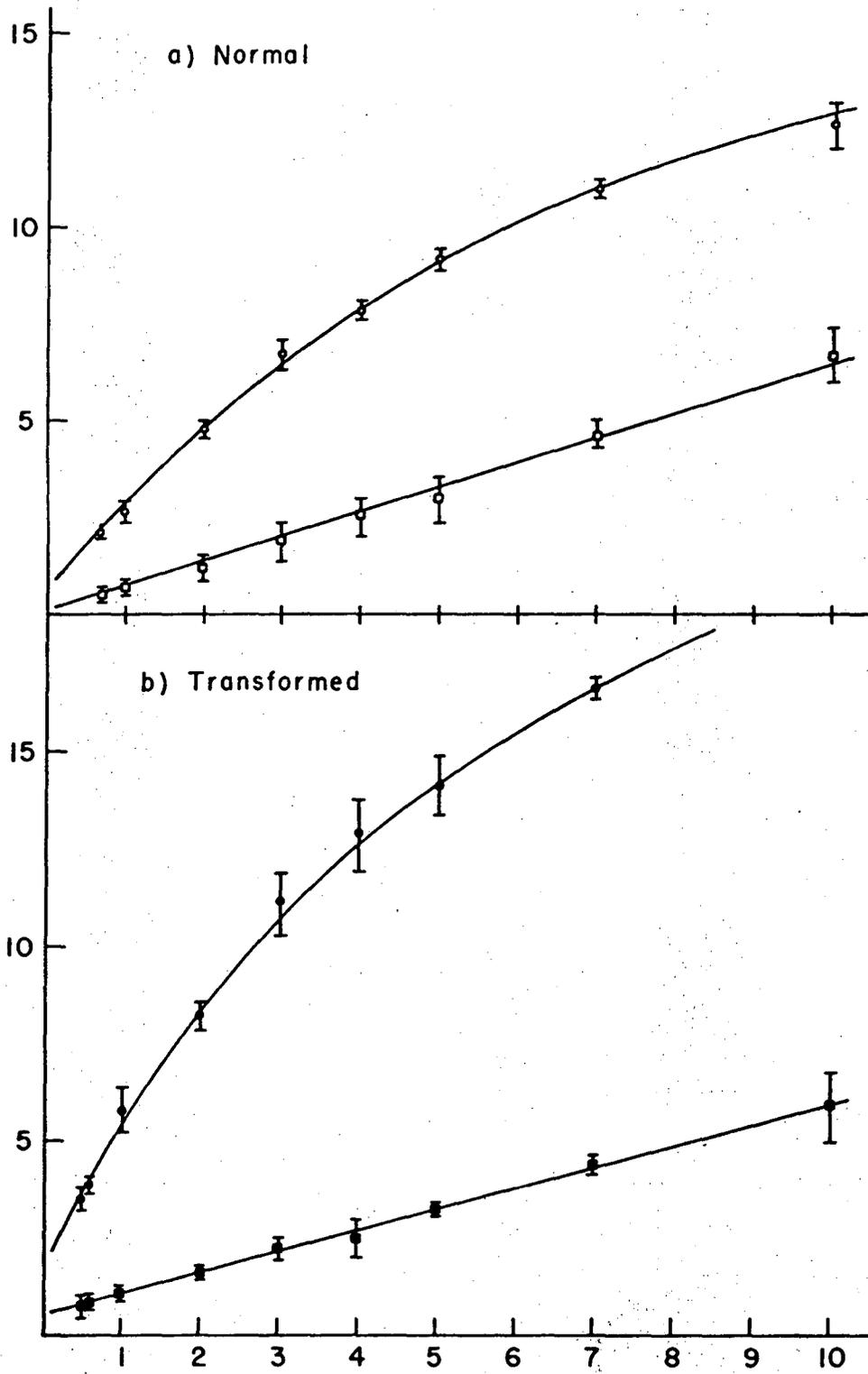


Fig. 2.

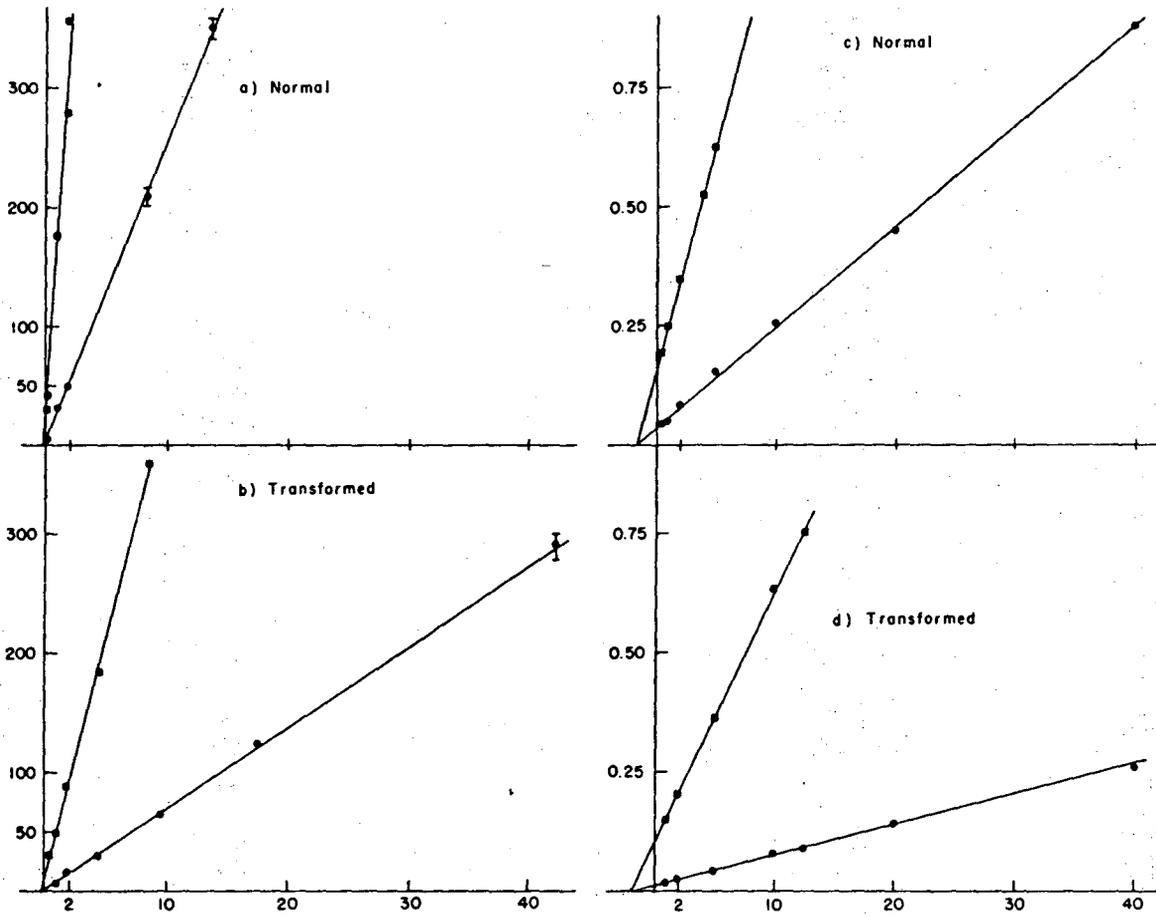


Fig. 3.

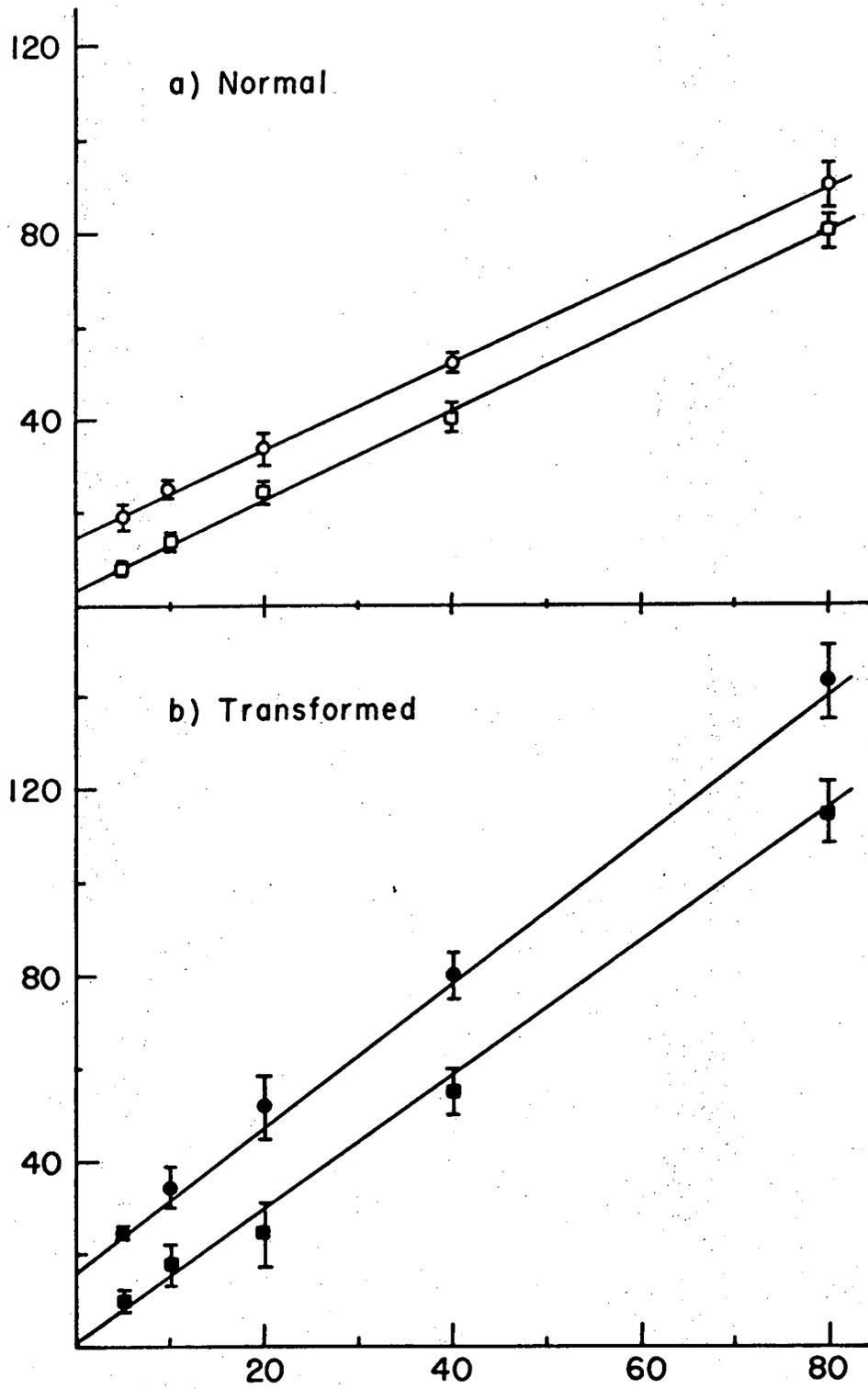


Fig. 4.

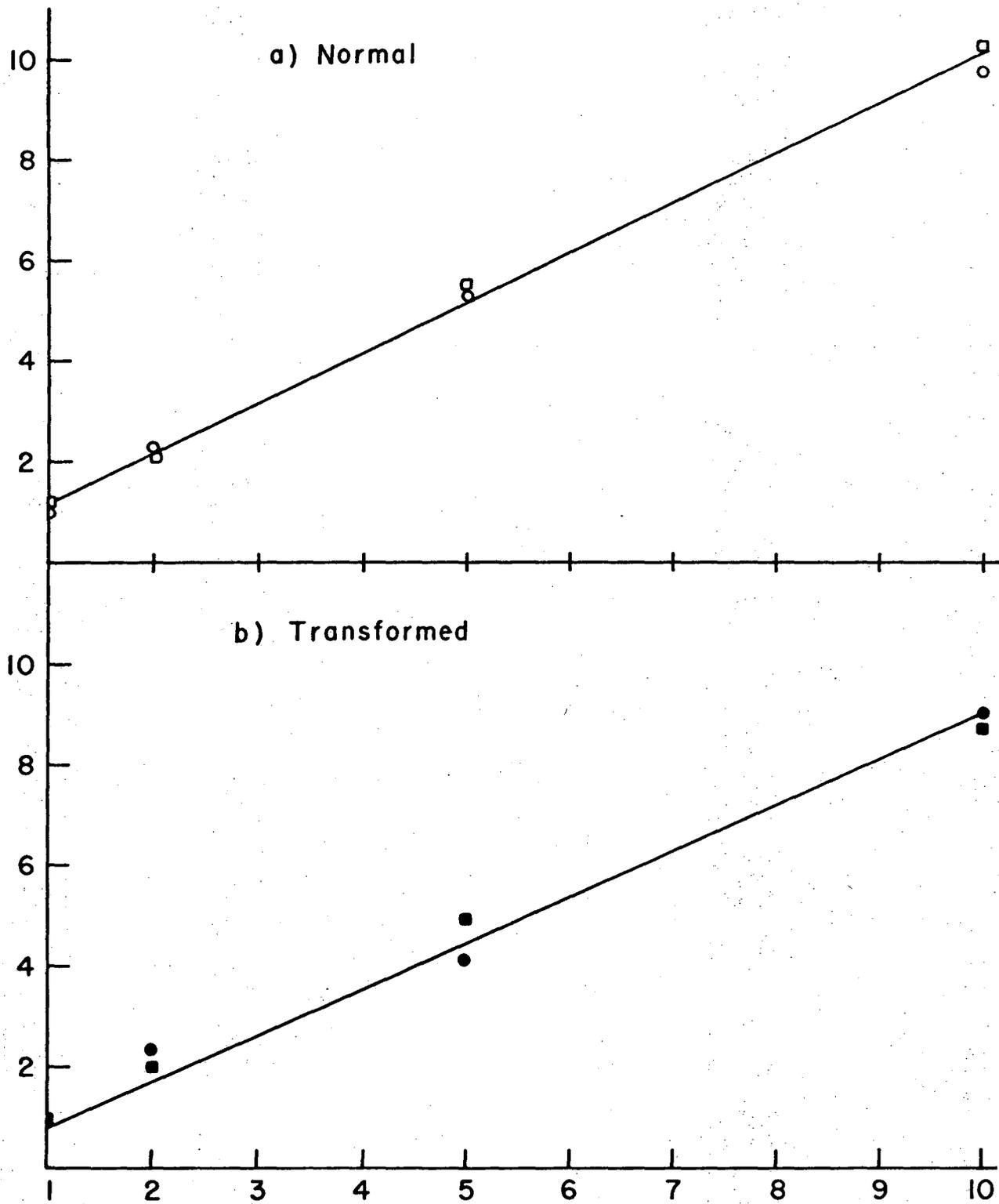


Fig. 5.

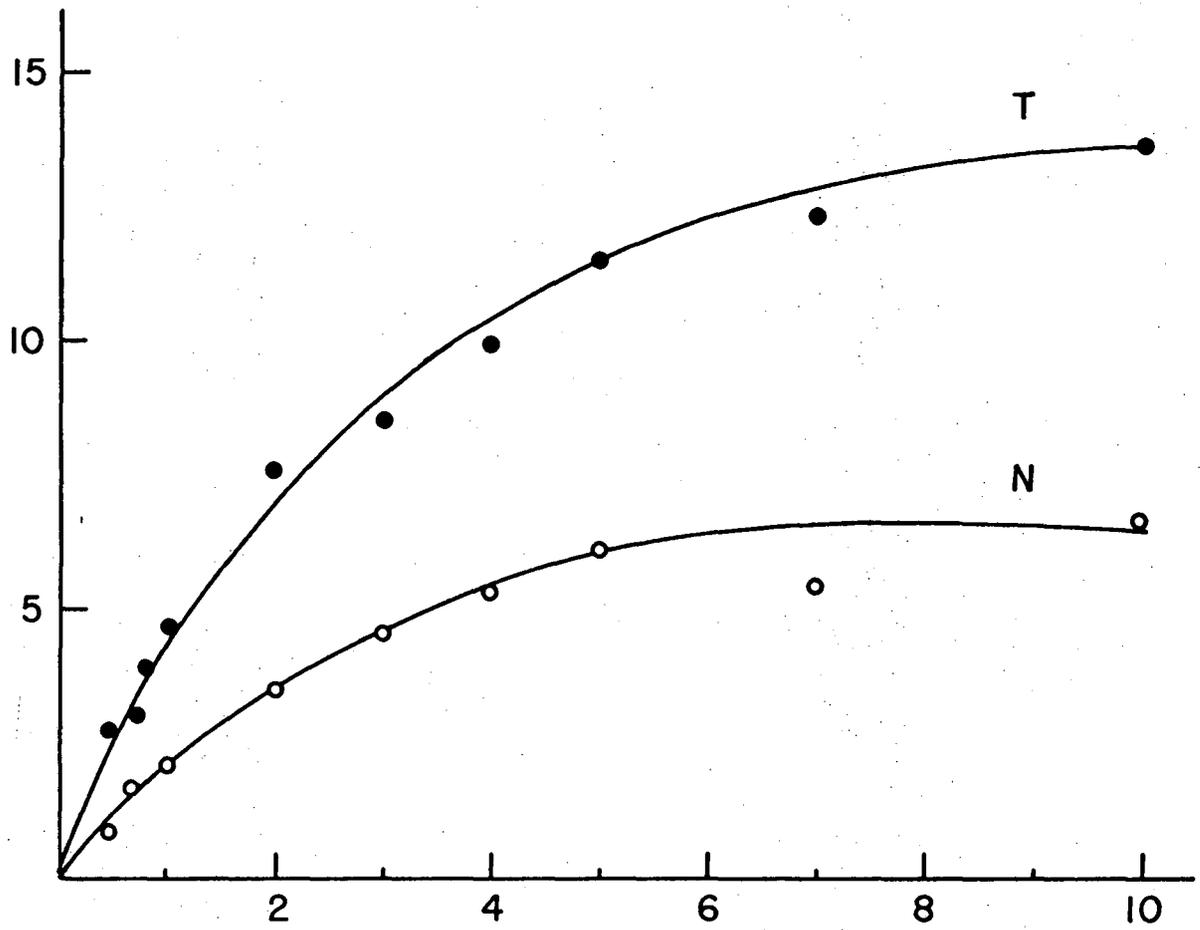


Fig. 6a.

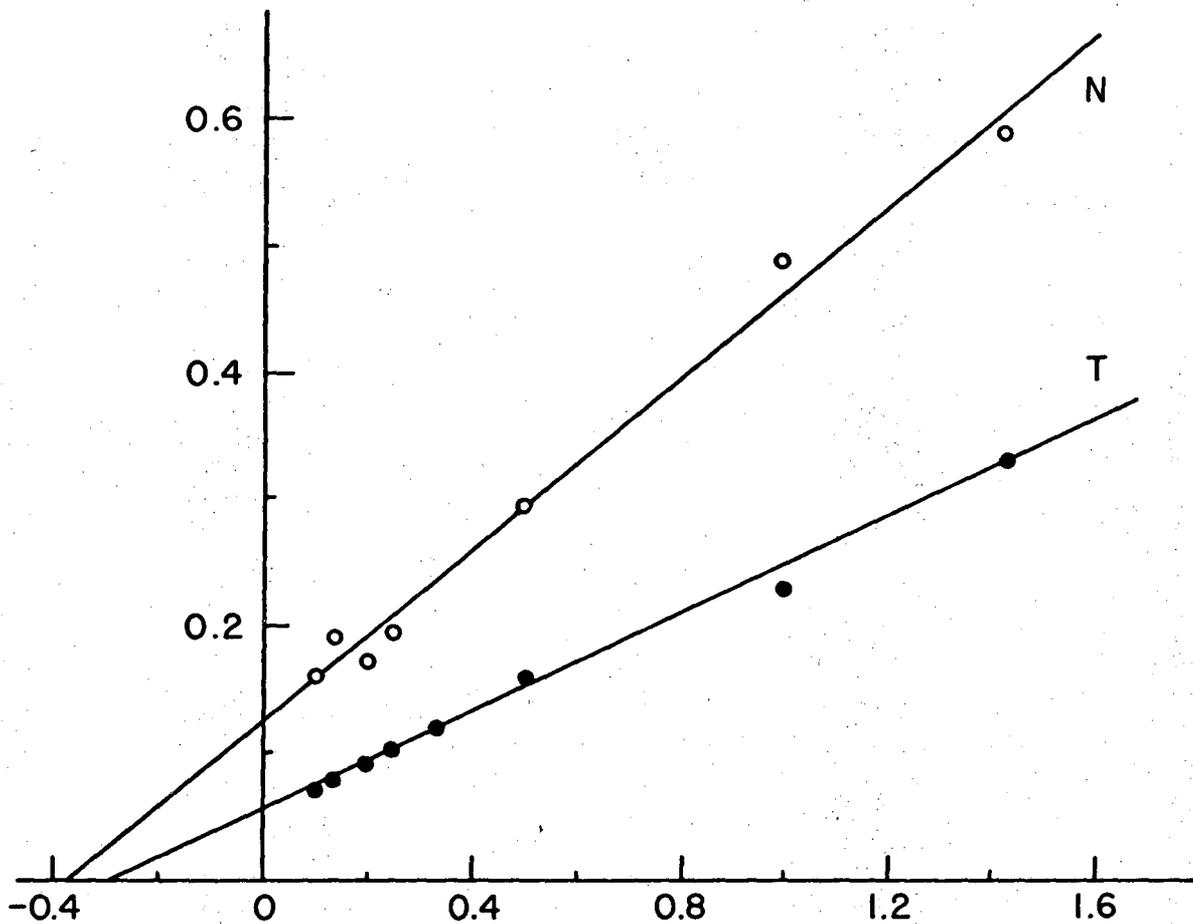


Fig. 6b.

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