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GROWTH FACTOR INTERACTIONS IN THE TISSUE CULTURE OF TUMOROUS AND NONTUMOROUS *NICOTIANA GLAUCA-LANGSDORFFII*[1]

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ABSTRACT

SCHAEFFER, GIDEON W., HAROLD H. SMITH and MARION PARTRIDGE (Brookhaven National Laboratory, Upton, N. Y.) Growth factor interactions in the tissue culture of tumorous and nontumorous *Nicotiana glauca-langsdorffii*. Amer. Jour. Bot. . Illus. 1963.--Tissues representing tumorous and nontumorous *Nicotiana glauca-langsdorffii* were cultured on high (5x) and low (1x) concentrations of a modified White's basic medium containing 2.9×10^{-6} M indoleacetic acid. The growth response of tissues of both the tumorous and nontumorous genotypes to supplements of kinetin, glutamine, inositol and nucleic acid constituents added singly and in all combinations were noted on high salt media. The nucleic acid components inhibited growth and were omitted from low salt media. The best growth response was observed with glutamine and inositol for tissues from the tumorous hybrid and with glutamine, inositol and

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kinetin in the nontumorous type. Kinetin was a distinct and consistent requirement for rapid growth of nontumorous tissues, but no appreciable kinetin effect could be observed with tissues from the tumorous genotype.

THE CULTURE of plant tissues in vitro has been utilized effectively in the study of growth, nutrition and differentiation. Tissue culture studies have contributed significantly to an understanding of differences in the growth requirements of normal and abnormal growth such as plant tumors. It has been clearly demonstrated that tumors induced in Vinca tissue by the crown-gall bacterium can be cultured readily without exogenous auxin and kinetin; thus they presumably autonomously synthesize enough of these substances for rapid growth (Braun and Wood, 1961). Plant hormones are among the principal requirements for the culture of many types of normal tissues.

Braun (1958) has shown that in addition to certain hormonal alterations progressive tumor transformation involves the apparent activation of distinct biosynthetic systems. Normal cells grown on White's basic medium and sucrose require for rapid growth auxins, kinetin, inositol, glutamine, asparagine, cytidylic acid and guanylic acid. Tumorous cells require only inorganic salts and sucrose. More recently Wood and Braun (1961) and Braun and Wood (1962) suggested that some of the growth factor systems are ion activated and changes in membrane permeability and ion-transport mechanisms are involved in the transformation of a normal cell to a tumor cell.

Certain interspecific combinations of Nicotiana are genetically predisposed to tumor formation (Smith and Stevenson, 1961). Among these hybrids are

N. glauca-langsdorffii and *N. suaveolens-langsdorffii* which form tumors spontaneously at flowering or early senescence. These tumors occur without the involvement of an external causative agent and do not normally occur during periods of rapid stem elongation.

A few years ago a nontumorous type of *N. glauca-langsdorffii* was obtained by Izard (1957) from x-rayed seeds of the hybrid. This nontumorous mutant of *N. glauca-langsdorffii* and the ordinary tumor-forming hybrid seemed to be excellent material to elucidate further the growth factor requirements and the interaction of growth factors in culture of tissues from tumorous and nontumorous forms of the same residual genotype. Recent studies (Schaeffer and Smith, 1963) showed that *N. glauca-langsdorffii* tissues representing the nontumorous type had a much more complex requirement for exogenous growth factors than tissues representing the tumorous type of the same interspecific combination. This suggested that tissue cultures from tumors induced by the crown-gall bacterium and *Nicotiana* hybrid tumors resulting from genetic instability have in common the capacity for growth without external supplies of substances, including kinetin-like compounds.

This report deals with the interaction of kinetin, glutamine, inositol and nucleic acid constituents in sterile cultures of tumorous and nontumorous tissue of *N. glauca-langsdorffii*. The effects of several inorganic salt levels upon growth of these tumorous and nontumorous tissues are also considered.

METHODS--*Tissue culture medium*--In the experiments reported here a modified White's basic medium (1954) including glycine and the vitamin was utilized. Iron was added as an ethylenediaminetetraacetic acid (EDTA) chelate and the

medium was supplemented with 1.1×10^{-3} M KCl as previously described (Schaeffer and Smith, 1963). The basic medium containing 0.75% Difco agar was supplemented with an organic fraction consisting of 5×10^{-4} M myo-inositol and 1×10^{-3} M L-glutamine, and additionally supplemented with 5×10^{-4} M cytidylic acid, 4×10^{-4} M guanylic acid, 5×10^{-4} M hypoxanthine, 2.3×10^{-6} M and 9.1×10^{-6} M 6-furfurylamino purine (kinetin), 2.9×10^{-6} M indole-3-acetic acid (IAA) and 2% sucrose. Cytidylic acid, guanylic acid and hypoxanthine will be referred to in this communication as the nucleic acid (NA) fraction. The variables in the experiments were: (1) high and low inorganic salt levels and (2) addition of glutamine, inositol and nucleic acid fraction singly and in all combinations in the presence and absence of kinetin. The nucleic acid fraction was omitted from the low salt experiments. Indole acetic acid was added to all media at 2.9×10^{-6} M.

Inorganic salt levels--The tumorous and nontumorous tissues of *N. glauca-langsdorffii* were cultured on modified White's medium with 2 levels of all the inorganic salts except iron chelate which was kept constant throughout the experiments. The high salt media were prepared by increasing all the White's basic medium salts, including the supplemented KCl, to a level 5x unit concentration.

In the experiments with high salt solutions all constituents except glutamine were added before autoclaving in the first and second transfer and all except glutamine and inositol in the third transfer. These growth factors were sterilized by Seitz filtration and aliquots added aseptically to individual culture flasks before agar solidification. The criterion for comparing growth

of tumorous and nontumorous types in the high salt experiments was the mean weight of tissues from 3 successive transfers on identical media. Each treatment was replicated 6-12 times in each transfer. The experiment was considered as a single replicate of a 6 factor experiment so that a reasonable estimate of the error variance in the analysis of variance was taken to be the pooled 4, 5, and 6 factor interactions. The 6 factors are genotypes, transfers, and the 4 growth factors kinetin, inositol, glutamine, and nucleic acid components.

The low salt solutions contained unit concentrations of White's basic medium supplemented with KCl and with versene chelated iron. In these preparations both glutamine and inositol were added aseptically. To measure and compare growth of tumorous and nontumorous tissues in the low salt experiments the mean weight of 10-12 replications from the second transfer was used. The data from the first low salt transfer are not reported here because the tissues showed considerable carry-over effect.

RESULTS--Of the 4 growth factors under consideration, the NA fraction was the only component which consistently decreased growth. It did not, however, appreciably affect the pattern of response of either tumorous or nontumorous tissue to glutamine and inositol. This result is illustrated in Fig. 1. One of the most striking features of these experiments is the pronounced growth response of nontumorous tissue to kinetin, particularly in the presence of glutamine alone. The largest growth responses by nontumorous tissue in the absence of kinetin were obtained with inositol alone or glutamine in the presence of inositol. Tumorous tissue clearly did not respond to kinetin; but, there was some response to glutamine and inositol.

The analysis of variance of the culture of tumorous and nontumorous types on 16 high salt media over 3 transfers showed large effects due to: (1) kinetin, (2) tumorous vs nontumorous genotype, and (3) the kinetin-genotype interaction. This is illustrated in Fig. 2-6. Large and significant sources of variation were noted for inositol, glutamine, and the glutamine-genotype interaction as well as for the kinetin-glutamine-genotype interaction. The NA effect, although significant, was due to inhibition of growth. The only other significant component of variance was due to transfers. This was probably dependent on a combination of several factors among which the following 3 may be important: (1) The kinetin concentration during the first transfer was 9.3×10^{-6} M; during the second and third transfers it was 2.3×10^{-6} M. It should be noted, however, that the effects of the 2 kinetin concentrations are very similar in the presence of 2.9×10^{-6} M IAA (Schaeffer and Smith, 1963). (2) Inositol was added to the media before autoclaving in the first and second transfer but added aseptically after autoclaving in the third transfer. (3) There may have been a real transfer effect due to cell selection with successive transfers or depletion of endogenous growth substances.

The response of the tumorous and nontumorous tissues to treatments with several growth factors in the low salt media was, in many respects, similar to that observed on the high salt media. There was, however, some indication of a differential salt-level effect. One indication was the mediocre growth response due to glutamine added singly and the pronounced growth enhancement by glutamine in the presence of inositol in the low salt experiments. This was particularly obvious for the tissues of the tumorous genotype. Similar glutamine and

glutamine + inositol treatments in the high salt experiments produced nearly equal growth. Another indication was a greater inhibition of growth at a low salt level due to kinetin in the rapidly dividing tumorous tissue in the glutamine + inositol treatment than was observed on the high salt media. Finally, there was a reduced growth rate and a very similar kinetin response of both tumorous and nontumorous tissues when cultured on nonsupplemented media (Fig. 7).

Growth rate is an important consideration in evaluating tissue responses to hormone treatment. For example, kinetin inhibited growth of rapidly growing tissues treated with glutamine and inositol, as shown in Fig. 7, whereas slow growing tumorous tissues on nonsupplemented media showed a growth acceleration due to kinetin. These responses were much more pronounced on low salt media; but similar responses of tumorous tissues grown on high salt media were indicated.

The most obvious treatment effects in this study are due to kinetin and glutamine. Inositol enhances growth and improves the condition and general appearance of the callus tissues; however, inositol enhances the growth of both the tumorous and nontumorous _N. glauca-langsdorffii_ tissue. A differential inositol requirement for tumorous and nontumorous tissues is not indicated under these culture conditions. This may simply reflect the stage to which normal cells have been transformed in the tumor genotype. On the other hand, kinetin on both the high and low salt media consistently increased growth of nontumorous tissues. The growth factor interactions seem to be of paramount importance. It is difficult, if not impossible, to separate a direct effect of glutamine and inositol from indirect effects due to hormone actions and interactions.

Since tumor formation is essentially a failure of organized differentiated growth and in the Nicotiana hybrids is conditioned by the genotype (Smith and Stevenson, 1961), it appears that there is an interference with the normal transfer and/or utilization of information from the genes to those cellular elements responsible for tissue organization. The tumorous hybrid genotype contains this information because it is composed of a combination of 2 genomes, each of which by itself produces normal plants of a recognized species. In an earlier communication it was postulated that IAA-kinetin treatments might elicit the production of cell substances in the hybrid which cause tumors or, in other words, interfere with genetic information or its transfer (Schaeffer and Smith, 1963). Recent studies by Henderson have shown that intermediate oxidation products are formed from 6-(substituted) aminopurines by xanthine oxidase. These intermediate products strongly inhibit the oxidation of purines by the enzyme. Henderson's findings support the contention that the IAA-kinetin interaction may involve the production of compounds that interfere with normal differentiation so as to induce plant tumor development. It may be possible to explain the auxin-kinetin interactions observed if future experiments show that kinetin or the oxidation products of kinetin also inhibit other oxidases, as for example IAA oxidase or ascorbic acid oxidase.

Experimental findings such as the IAA-kinetin interaction in the induction of tumors in seedlings of N. suaveolens-langsdorffii (Schaeffer, 1963) and the auxin-kinetin interaction in the culture of tumorous and nontumorous tissue of Nicotiana (Schaeffer and Smith, 1963) also suggest the elaboration of cell constituents that interfere with and alter normal control.

The results reported here with the genetically conditioned hybrid system in general corroborate the results from plant tumor studies with the crown-gall microorganism. On the basis of growth factor requirements, particularly for glutamine and inositol, it appears that the degree of tumorization of *N. glauca-langsdorffii* may correspond to cells partially transformed by bacteria rather than fully transformed tumor cells. This is indicated by the increase in growth obtained with tumorous tissue cultured with glutamine + inositol on low salt media. Such a response would not be expected for fully transformed crown-gall tumor cells (Braun, 1958); but may, on the other hand, simply reflect differences between the growth requirement of *Nicotiana* and *Vinca* cells.

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Fig. 1. Effect of kinetin, glutamine, inositol and nucleic acid components added singly and in all combinations upon the culture of tissues from tumorous and nontumorous _N. glauca-langsdorffii_. Histograms represent means from 3 transfers with 6-12 replications/transfer. Tissues were cultured for 34 days/transfer on modified White's basic medium with 5x unit inorganic salt concentration and 2.9×10^{-6} M indole-3-acetic acid.

Fig. 2-6. Graphic representation of growth factor interactions in the tissue culture of tumorous and nontumorous _N. glauca-langsdorffii_. F values and level of significance from analysis of variance are indicated alongside brackets showing effects and interactions.

* indicates significance at 5% level.

** indicates significance at 1% level.

*** indicates significance at 0.1% level.

Fig. 2. [T] genotype (tumorous vs nontumorous), [K] kinetin, [KT] kinetin-genotype interaction. Fig. 3. [G] glutamine, [GT] glutamine-genotype interaction. Fig. 4. [KG] kinetin-glutamine interaction, [KGT] kinetin-glutamine-genotype interaction. Fig. 5. [I] inositol, [IT] inositol-genotype interaction. Fig. 6. [IG] inositol glutamine interaction.

Fig. 7. Effect of kinetin, glutamine and inositol added singly and in all possible combinations upon cultures of tissue from tumorous and nontumorous _N. glauca-langsdorffii_. Histograms represent means ± standard error of 10-12 replications from a single transfer. Tissues were cultured for 34 days on modified White's basic medium with unit inorganic salt concentrations and 2.9×10^{-6} M indole-3-acetic acid.

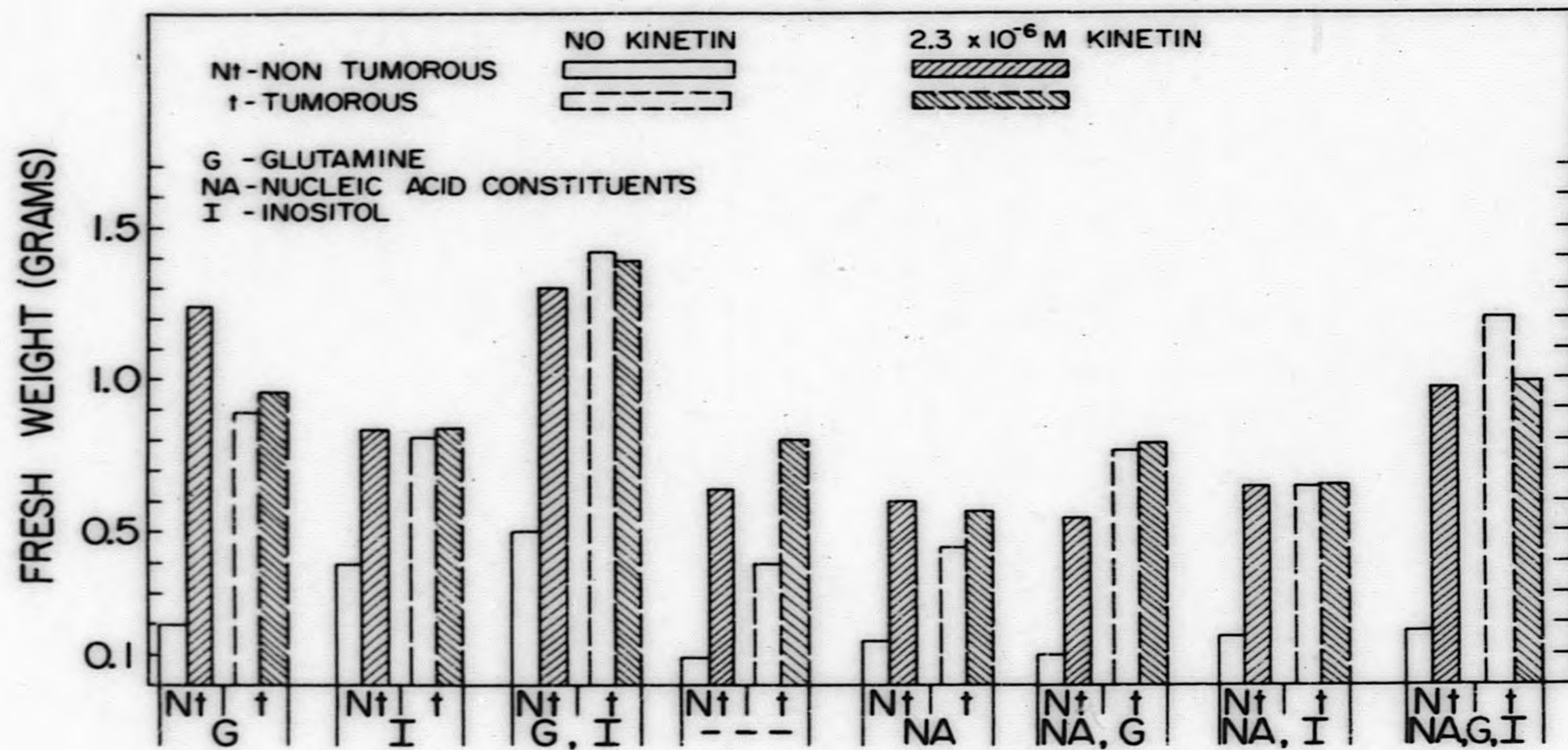

FIGURE 1

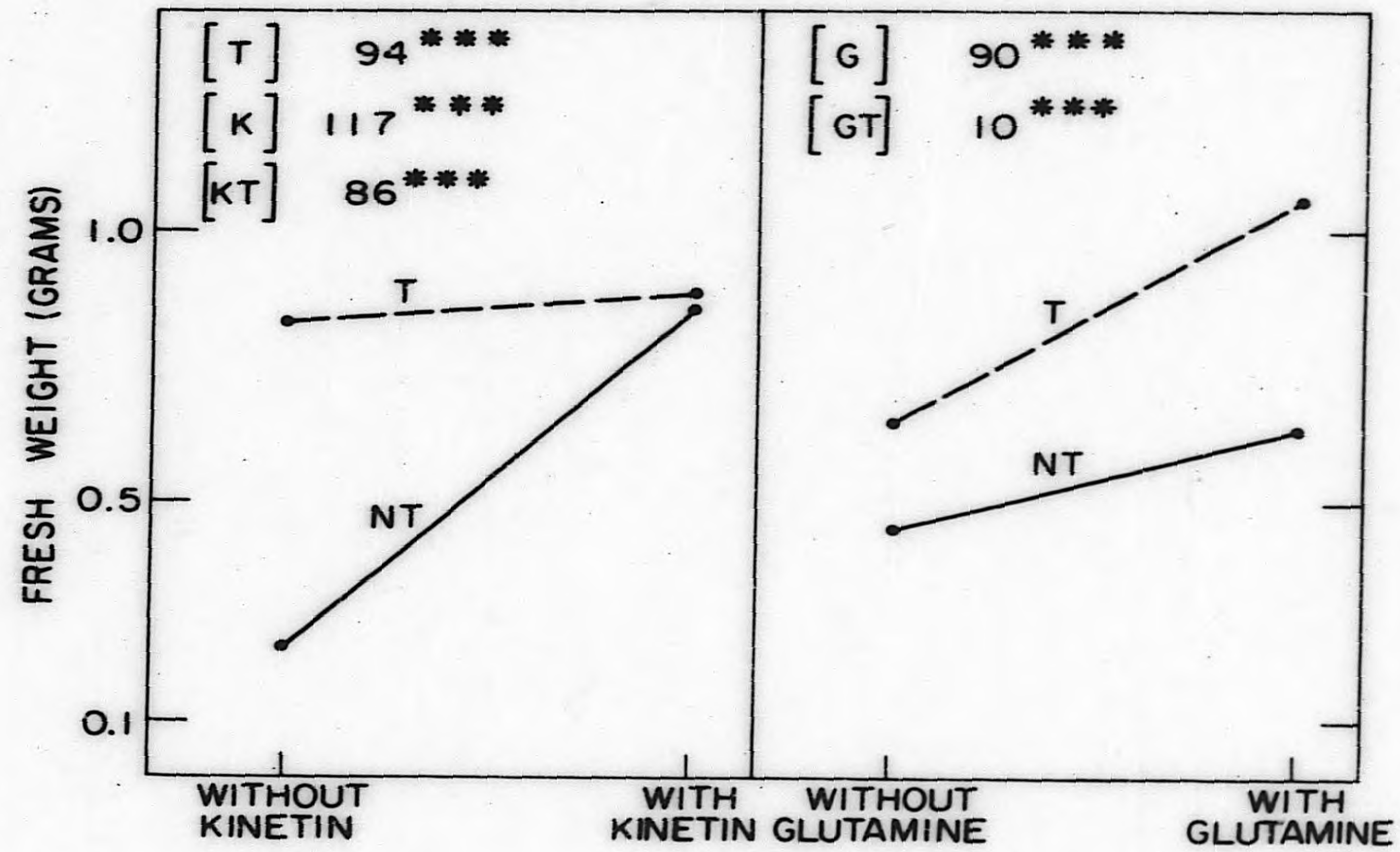

FIGURE 2

FIGURE 3

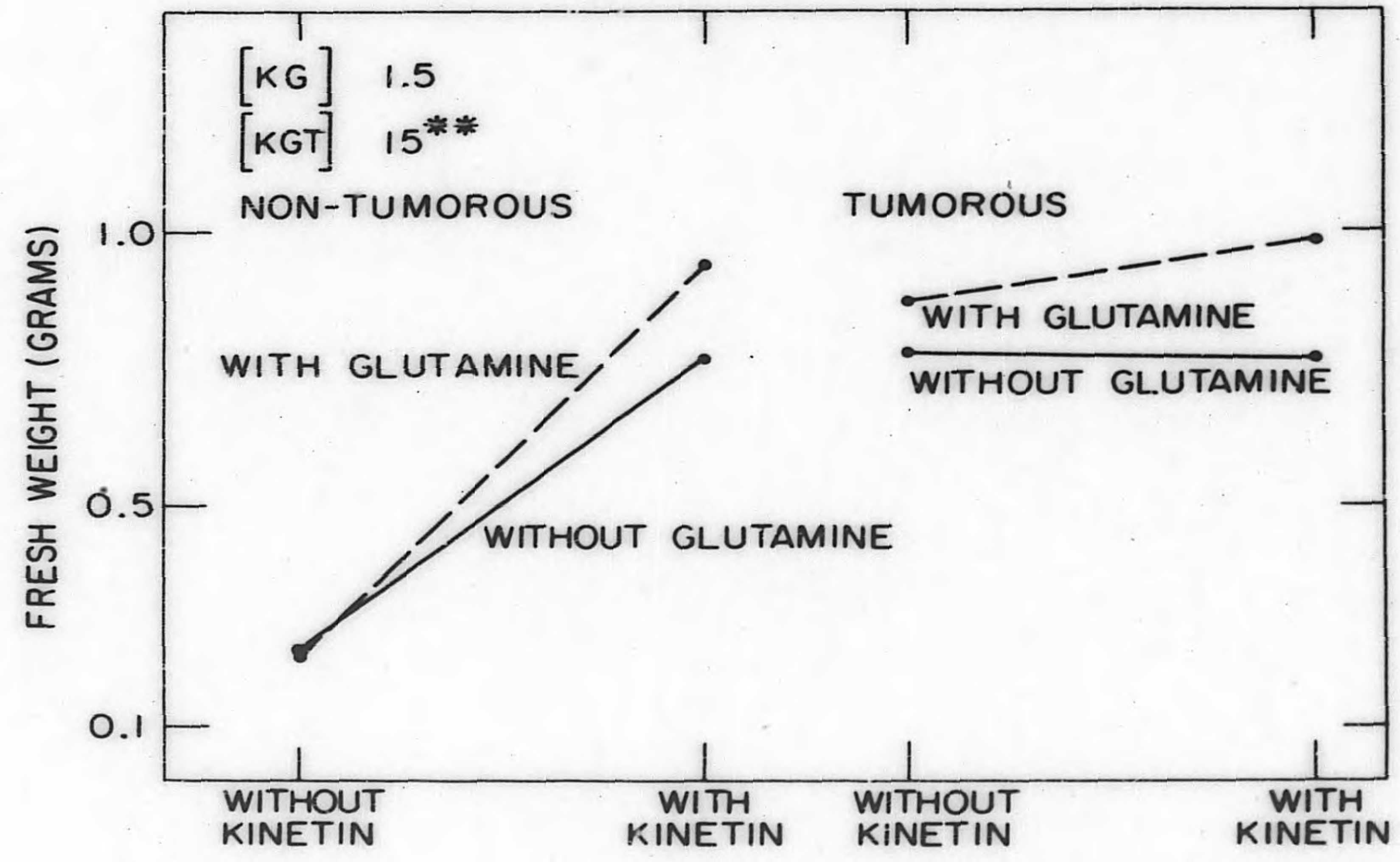

FIGURE 4

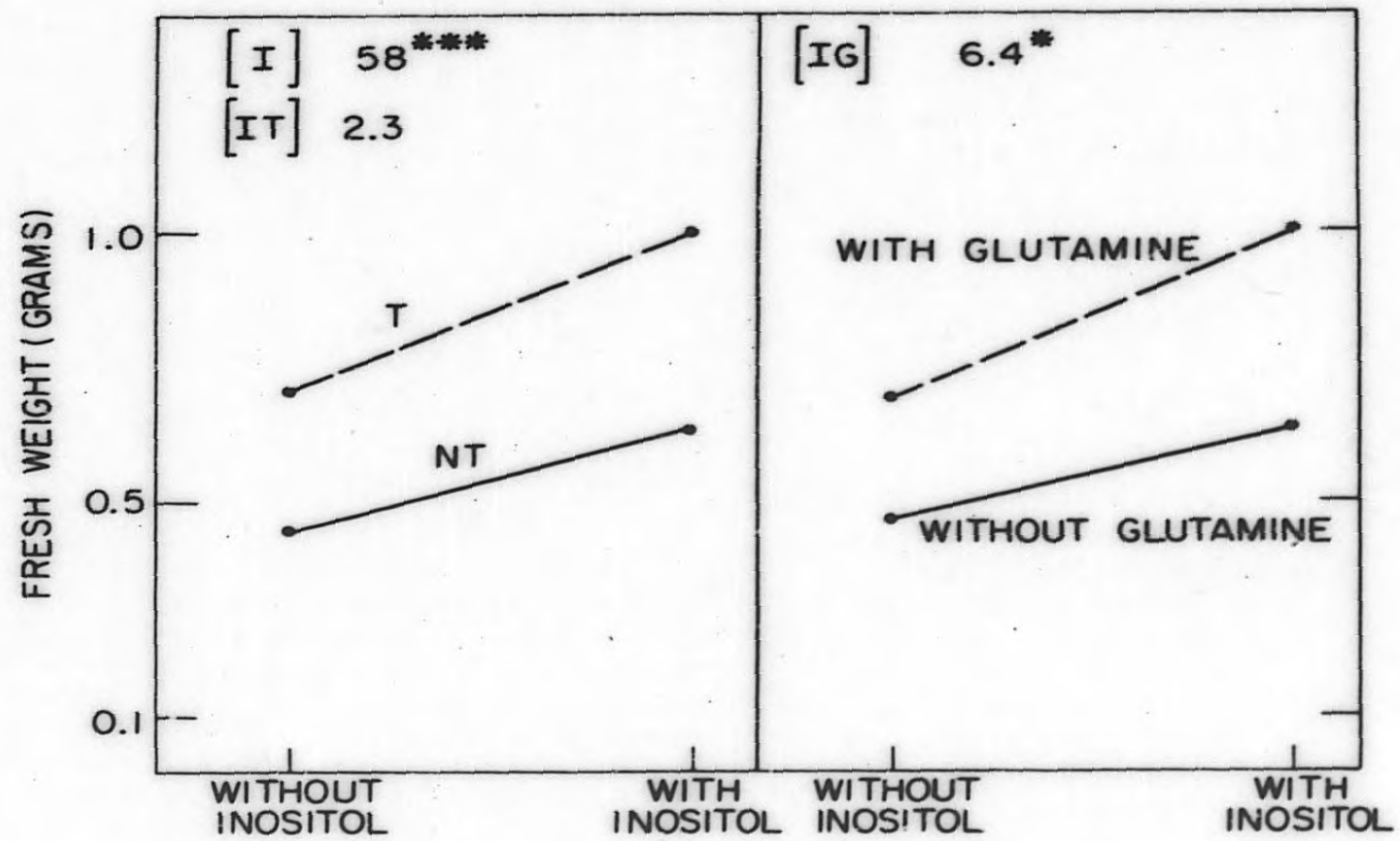

FIGURE 5

FIGURE 6

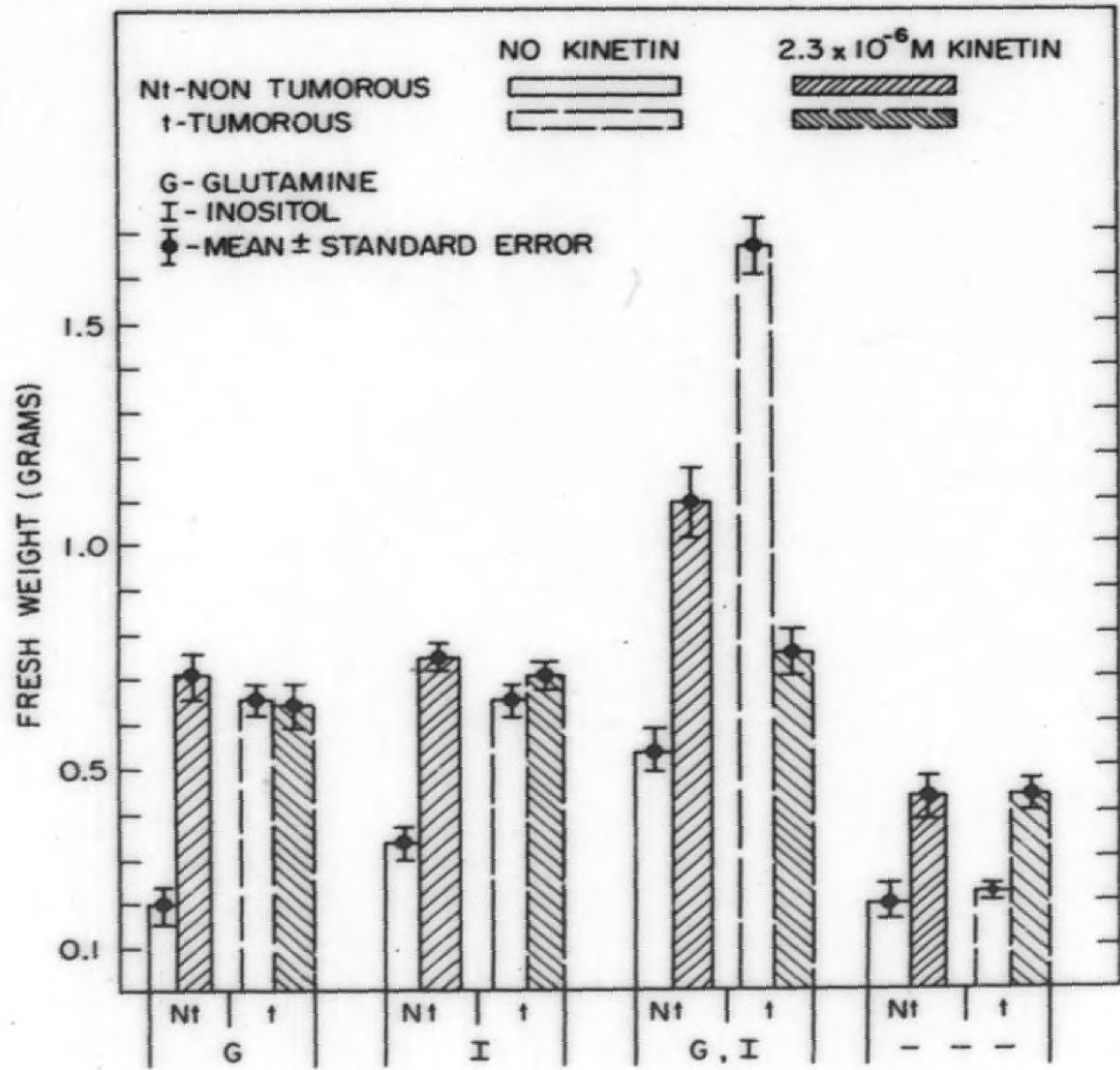

FIGURE 7

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