STUDIES ON THE METABOLISM OF SOME ACTINOMYCETES ASSOCIATED WITH POTATO SCAB

by

ROBERT JOHN DOUGLAS

A THESIS

Submitted to the School of Advanced Graduate Studies of
Michigan State University of Agriculture and Applied Science
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1955

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ABSTRACT OF THESIS

A number of experiments were carried out in order to obtain a more complete knowledge of the metabolism of the potato scab organism, Streptomyces scabies. Experimental techniques were described for the preparation of mycelial homogenates for manometric investigations; the effect of such variables as the amount of inoculum, mycelial age, and the influence of starvation was determined. Homogenates were shown to be capable of oxidizing a number of metabolically important organic acids and amino acids. Difficulty was encountered in the demonstration of α-ketoglutarate oxidation, and pronounced lags were noted before the maximum oxidative rate was attained with several compounds (e.g. succinate, tyrosine, phenylalanine). The possibility of membrane impermeability to the added substrate was discussed.

The R.Q. of the homogenates metabolizing stored reserves was found to be $\approx$0.86. This value, coupled with the observation that NH_3 was liberated during autorespiration, was interpreted as indicating that the endogenous activity was supported, at least in part, by nitrogenous reserves.

The oxidations of glucose, glutamate, and succinate were shown to be incomplete. By the addition of dinitrophenol to starved homogenates the amount of glucose oxidized was considerably increased. This was believed to be the result of the inhibition of assimilation by dinitrophenol.

Pathogenic cultures were shown to cause pronounced

pigment formation in a peptonized milk medium. Such cultures catalyzed the oxidation of tyrosine and dihydroxyphenylalanine with the formation of melanin.

Extracts obtained from mycelium disrupted by grinding with alumina were unable to bring about an exogenous oxygen uptake in manometric experiments. These extracts contained dehydrogenases which could be demonstrated using the dye 2,6-dichlorophenol indophenol. The addition of DPN was shown to stimulate dye reduction in the presence of glutamate, malate, and fumarate.

Mycelial homogenates and extracts were able to form glutamate in the presence of α-ketoglutarate and an amino acid (aspartic, alanine, leucine, or valine). The aspartic-glutamic transaminase activity was the highest of those observed. No evidence was obtained for a direct transamination between α-ketoglutarate and the amides asparagine and glutamine although both compounds were deaminated. Difficulties were encountered in the demonstration of transamination because of high glutamate "contamination" and non-specific amino acid formation.

INTRODUCTION

The scab disease on potatoes has caused considerable economic loss by virtue of lowering the potato grade or in extreme instances rendering the tubers unfit for sale. Attempts to control the disease by soil amendments or cultural practices have been disappointing probably because the organism responsible appears to be a natural soil inhabitant. Conditions which render the soil unfavourable to the growth of the scab organism generally also seriously interfere with other members of the microbial population, in which case deleterious effects on soil structure and fertility may become apparent. In occurrence, the disease is widespread although there is evidence of regional variation in its severity; certain soils are considered to have a high scab potential.

Over the past fifty years an appreciable amount of research has been directed to the scab problem. Individual studies have dealt with: the disease process as such (83), possible methods of prevention (1), and the physiology of the organisms concerned (102). The present work is concerned with the latter aspect of the problem and observations are reported on some of the metabolic activities of scab-producing actinomycete isolates. The individual sections which follow describe the respiratory activities of mycelial homogenates, pigment formation of the cultures, dehydrogenase reactions of cell extracts, and the ability of actinomycete preparations to carry out transamination reactions.

LITERATURE REVIEW

In the study of the respiration of both actinomycetes and filamentous fungi most workers have used cellular material harvested from shake cultures (79). Agitated cultures of this type grow much more rapidly (23) and, in addition, the formation of aerial mycelium is retarded. Aerial mycelium may be metabolically different from vegetative hyphae (79) so that a mixture of the two may give a confusing picture of the metabolism of the organism. It has been noted, however, that the reducing conditions which occur in submerged culture can give rise to an accumulation of intermediate metabolites which would not appear on surface culture (79).

Considerable work has been done on the nutrition of the streptomycetes, especially those concerned with antibiotic production (79). Waksman (103) noted that peptic digests of casein were good nitrogen sources for these organisms. Ammonium salts supported the growth of Streptomyces fradiae, but no antibiotic was formed. Bennett (9) reported that complex nitrogenous compounds were also required to produce good antibiotic yields with Streptomyces griseus. Nitrate was used as a nitrogen source by Streptomyces coelicolor, but good growth was obtained only when precautions were taken against the development of excess acidity (24). The addition of yeast extract to nitrate-medium, however, considerably reduced the time required to obtain maximum cell yields. Gottlieb (49) concluded that the actinomycetes could utilize

various carbohydrates, alcohols, fatty acids, and protein compounds as carbon sources, although some strains differed in their ability to utilize disaccharides apparently because of the lack of specific hydrolases. The data of Nickerson and Mohan (75) indicated that while S. fradiae could obtain both carbon and nitrogen from glutamate, the addition of glucose to the medium resulted in much greater growth (approximately two fold). Cochrane and Dimmick (23) pointed out that a "balance" between carbon and nitrogen supply was a prime requirement for the successful cultivation of S. coelicolor. The work of Umbarger (99) indicated that the nutrient medium appeared to exert a considerable influence on the enzymic potential of Escherichia coli.

The phases of growth observed in a bacterial culture are usually not seen with actinomycetes although if growth is retarded by reducing the temperature then distinct phases of growth may become apparent (49). A number of authors indicated that the growth rate was variable during the development of a culture (49, 50, 79), but little has been done to demarcate discrete phases of growth. Emerson (36) showed that with Neurospora crassa a growth phase existed comparable to the log phase in unicellular organisms. Assuming that apical growth occurred, and that in submerged shake cultures the spherical masses would grow in three planes, this worker was able to show that, for a time at least, a plot of the cube root of dry weight against time gave a straight line relationship. For such a period (up to 50 hours for this organism)

a phase analogous to the exponential phase probably existed.

A number of investigators have indicated that young and mature mycelium may differ considerably in physiology (50, 79). Rolinson (84) showed that both the R.Q. and the Q_{O_2} values obtained with Penicillium chrysogenum changed markedly as the culture aged from 24 to 96 hours. It was also noted that the degree of aeration during the early stages of growth exerted a considerable influence on the nature of the respiratory enzymes formed. Gottlieb (49) compared actinomycete growth with that of the fungi emphasizing the fact that the cellular material, hyphal in nature, may be actively growing at the apical portions and yet be quite static or even dead in older parts of the mycelium. This author noted that O_2 consumption did not parallel growth, and interpreted this observation as indicating a non-uniformity in physiology throughout the cellular mass. Gottlieb and Anderson showed that with S. griseus respiratory activity declined after the initial 12 to 15 hour incubation period and that Q_{O_2} values fell to only 25 percent of maximum at 48 hours (50). They suggested that an "inherent change in respiration" occurred as the cells aged. This latter observation could probably be explained, in part, by the suggestion that older portions of the mycelium, be they inactive or dead (49), while not contributing maximally to oxygen uptake, still influenced the nitrogen or dry weight basis upon which the respiration data were based. The consensus of opinion appears to favor the use of young material where high respiratory activity is desired (49, 50, 79).

Cochrane demonstrated that the respiratory activity of 4-day cells of S. coelicolor was higher than that of 2 or 3-day cells (28). He interpreted these findings on the basis of cellular permeability, and suggested that a permeability increase in the older cells was responsible for the apparent increase in respiration. In some cases the choice of a particular cell age has been determined by a compromise between usable quantities of mycelium and residual respiratory activity (31).

Erikson and Webley (38) and Webley (104) reported observations on the respiratory activities of a thermophilic actinomycete Micromonospora vulgaris. These authors grew the organism in still culture for 2 days, and then attempted to separate the aerial and vegetative hyphae. While it is difficult to compare data from aerated and still cultures, incubation periods of 48 hours for thermophilic forms could reasonably be expected to produce cellular material which was physiologically mature, and which, by analogy to other work, had relatively low respiratory activity. These authors also reported that replicates showed considerable variation, and suggested that the complex organization of such an organism was partly responsible for the difficulties encountered. Some of the experimental techniques described (e.g. growing the organism on cellophane and washing the growth into respiration vessels) indicate the very real technical difficulties encountered with these forms. Webley (104) reported that the respiration of the aerial mycelium of M. vulgaris was inhibited

by an atmosphere of pure oxygen, and indicated that this may have been the result of inactivation of -SH containing enzymes. The latter were shown to be present on the basis of inhibition by iodoacetate and p-chloromercuribenzoate, although this inhibition could not be reversed by glutathione.

Nickerson and Sherman observed that the growth of Bacillus cereus could be converted to a filamentous type by cultivation of the organism in a medium low in Mg^{++} ions (76). They showed that the lack of Mg^{++} did not inhibit growth (the amount was equal to that observed in the complete medium) but appeared to inhibit cell division selectively. The respiratory pattern of the filamentous type cells was comparable to that of the fungi in that respiration was not greatly elevated (two to three fold) above the level of endogenous by the addition of substrate, in normally dividing cells the exogenous rate was about ten times that of endogenous. A further difference was noted in that normal cells showed a greater reduction in respiration in the presence of iodoacetate and from these data the authors concluded that the two forms differed with regard to the iodoacetate-sensitive portion of the oxidative system.

The respiratory activities of fungi and actinomycetes are comparable in that the endogenous respiration is usually very high and relatively constant over a considerable period of time (27, 59, 75, 91). This high endogenous respiration and failure to demonstrate a marked increase in oxygen uptake in the presence of exogenous energy sources led some

workers to conclude that the terminal respiratory system was working at "full-load" and that the failure to observe an increase was due to this limiting factor (77). Darby and Goddard (31), working with the cellulose decomposing fungus Myrothecium verrucaria, also reported high endogenous respiration values and noted that exogenous respiration could only be demonstrated by starving the mycelium in a salts-solution in the absence of nutrients so that endogenous respiration was depressed (presumably because of a decrease in stored reserve materials). Analogous starvation procedures have been frequently employed in an effort to show the oxidation of added substrate (35, 44, 55, 77, 78). Cochrane and Peck (29) stated that prolonged aeration of mycelial suspensions reduced the endogenous respiration of S. coelicolor but that such treatment reduced exogenous uptake by a like or even greater amount; a similar conclusion was reported with dermatophytes (10). Stout and Koffler reported that starvation reduced the dry weight of P. chrysogenum (91); shaking such starved mycelial suspensions in the presence of glucose led to a reincrease in dry weight due to oxidative assimilation. By the use of a staining technique (preliminary periodate oxidation followed by treatment with decolorized fuchsin) they were able to show the accumulation of polysaccharide-like reserves in the mycelial strands. The amount of glucose assimilated by mycelial suspensions represented a considerable portion (70 to 79 percent) of the glucose which disappeared from the shake flasks. On the basis of manometric

data, Darby and Goddard calculated that up to 90 percent of the glucose added to the reaction flasks was assimilated, and thus did not contribute to a measurable increase in O_2 uptake (31). It is tempting to try to explain the failure of Oginsky et al (77) to demonstrate appreciable exogenous respiration, even over 6 hour periods, on this basis of reserve formation by assimilation.

In the interpretation of manometric data the difference between exogenous and endogenous oxygen uptake is generally assumed to be the result of oxidation of the added substrate. Such an assumption may not be justified especially with forms in which endogenous activity is high (13). Using radiocarbon labelled cells, Cochrane and Gibbs showed that radiocarbon in the respired CO_2 of S. coelicolor was not reduced by the presence of added substrate (25); thus they concluded that the subtraction of endogenous respiration was justified. Similar techniques carried out with P. chrysogenum (13) indicated that radiocarbon in the respired CO_2 was not reduced during glucose oxidation, but was reduced during the oxidation of acetate. A different experimental approach was described by Wilner and Clifton (107) and Stout and Koffler (91). These workers showed that on the subtraction of endogenous respiration from the total oxygen uptake in the presence of a range of concentrations of added substrate, the data pointed to a relative constancy in the percentage oxidation which occurred. This was interpreted to indicate that no depression of endogenous oxygen uptake occurred in the presence of added

oxidizable substrate. Since the effect on endogenous respiration varies with the substrate and probably with the tissue involved (101), it would appear prudent to observe and record endogenous activity for each tissue preparation studied.

The products of the oxidation of carbohydrate by actinomycetes are thought to be CO_2 and H_2O although the results of Cochrane indicated that intermediate products occurred at least in some species (26). With S. coelicolor succinic acid was found to accumulate in considerable amounts. The mode of formation of this compound is of considerable interest in that although the key reactions indicative of a Kreb's cycle were shown to occur with this form (28), further work indicated that the accumulation of succinate resulted from the carboxylation of pyruvate and subsequent reduction of the product to succinate (26). Thus, while a tricarboxylic acid cycle was potentially available, the accumulation of one of the key compounds (namely succinate) apparently did not arise through this cycle. Other studies indicated that a conventional Embden-Meyerhof-Parnas glycolytic scheme plus Kreb's cycle did not operate, and indeed for S. griseus, Garner and Koffler (43) indicated that only the pyruvate and succinate oxidizing enzymes were constitutive - the other enzymes involved in the Kreb's cycle were considered to be adaptive. A conventional succinic dehydrogenase system was said to exist only where the organism was grown on succinate-containing media. A later report by Koffler (64) indicated that, by the use of cell-free extracts, the essential reactions of

the Kreb's cycle could be shown for S. griseus. Further confirmation of the operation of a Kreb's cycle in S. griseus has recently appeared (15, 30, 46). The work of Nickerson and Mohan (75) with S. fradiae showed that dehydrogenase activity against Kreb's cycle intermediates could be detected by dye reduction methods using cell extracts. These workers found that the E_o of the indicator dye was important in that dehydrogenase activity could not be demonstrated with methylene blue but that by using a 1:3000 solution of 2,6-dichlorophenol indophenol dye reduction was observed. Dehydrogenase activities for citrate, α-ketoglutarate, malate, pyruvate, lactate, and succinate were found. It is of interest to note that they were unable to demonstrate exogenous O_2 consumption manometrically using whole cells and they postulated that the organism assimilated the carbon source and then metabolized endogenously from a metabolic pool. They also reported that endogenous respiration was high and relatively constant over long periods of time in contrast to that of bacteria and yeasts. Experiments were discussed indicating that the actinomycetes were able to reduce triphenyltetrazolium chloride, and this observation in conjunction with the work of Brodie and Gots (14) would indicate the existence of a flavoprotein H-transport system, at least in this organism. Cochrane and Peck (28) stressed that the high endogenous respiration of intact cells could be avoided by the use of cell-free preparations. By this approach they were able to show exogenous respiration of citrate and α-ketoglutarate,

two compounds for which unequivocal O_2 uptake could not be shown with intact cells. Later work by Cochrane (29) indicated that a pentose shunt system functioned in some actinomycetes and that this pathway of hexose degradation accounted for a considerable portion of the glucose utilized. Analyses of the fate of labelled glucose during oxidation by P. chrysogenum previously mentioned (63) were also consonant with this scheme of carbohydrate degradation.

Preparation of Homogenates and Techniques

The study of filamentous organisms is difficult in that conventional manometric techniques are not applicable without some modification (31, 38). Various authors have "blended" or homogenized mycelium in order to make the respiring suspension more homogeneous (31, 72). Failure to do this results in poor agreement between replicates and makes the interpretation of exogenous O_2 uptake difficult (103). Darby and Goddard (31) showed that prolonged maceration of the mycelium of M. verrucaria had a deleterious effect on the respiratory activity of the mycelium. Their data are subject to criticism, however, especially with regard to the data reported from the use of a glass homogenizer. They remarked that on disruption "bleeding" could occur from fragmented mycelial strands and yet they washed the mycelium after disruption. The decrease in respiration of such preparations could readily be occasioned by the loss of enzymes and/or cofactors into the wash liquid and not be

caused by the homogenization procedure. On theoretical grounds it would appear that washing should be carried out prior to cell disruption.

The effect of the amount of inoculum on the character of the growth obtained has been investigated by Darby and Goddard (31). These workers showed that small pellets of mycelium were produced in shake cultures when large numbers of spores were introduced, whereas, a smaller number of larger mycelial masses resulted when the spore inoculum was decreased. They also commented that small pellets were desirable in that the rate of O_2 diffusion into large cell masses could be a limiting factor in the respiration rates obtainable.

Preliminary starvation procedures have been discussed, and the results appear conflicting. Certainly a decreased endogenous respiration rate is desirable; however, if this can be achieved only at the expense of a proportional or greater than proportional decrease in exogenous oxidation (10, 28), then such a procedure is of questionable value. Work mentioned before (31, 91) indicated that oxidative assimilation by fungi may account for more of the glucose lost from the reaction mixture than does oxidative degradation. Such a preferential conversion to reserve material is in agreement with the concept of utilization advanced by Nickerson and Mohan (75).

The biological activity of organic acids is dependent upon the pH of the system, and Simon and Beevers (89) noted

that the difference in activity of the anion and undissociated forms was such that statements of activity were relatively meaningless unless accompanied by data concerning the hydrogen ion concentration at which the effect was observed. With Kreb's cycle intermediates permeability effects may be of considerable significance (100); the work of Cochrane and Peck (28) has indicated this with regard to the oxidation of citrate by S. coelicolor. The use of cell free methods is of considerable value in overcoming permeability difficulties (28, 82, 100).

The foregoing summarizes a number of investigations which show:

1) the similarity of fungi and actinomycetes with regard to their respiratory activities,
2) the heterogeneous nature of mycelial growth and variation in metabolism upon aging of the cells,
3) some of the methods which have been used to grow cellular material and prepare it for respiration work,
4) the possibility of demonstrating dehydrogenase reactions with cell extracts.

SOURCE AND IDENTITY OF CULTURES

The cultures used in this work were originally isolated from scab lesions on tubers obtained from various parts of Ontario. Those designated with a prefix P have been shown to be capable of causing typical scab lesions in greenhouse infection trials against the variety Katahdin. These parasitic strains represent a rather homogeneous group with regard to gross appearance, biochemical activity (102), serological reactions (34), and phage sensitivity (74).

There is still considerable confusion as to whether one species or a group of species is responsible for causing potato scab (34). The taxonomy of the actinomycetes is unsettled (47), and the potential variability of these organisms adds to the problems involved (86). Criteria used in classification are vague in many instances although a recent report on carbohydrate utilization studies shows considerable promise (8). At present, there is some justification for considering those organisms capable of causing scab to be strains of Streptomyces scabies as opposed to those isolates unable to cause the disease.

GENERAL CONDITIONS FOR MAINTENANCE AND GROWTH

Cultures were maintained on slants of a synthetic glucose-asparagine agar (34). For inoculation of liquid media, spores were scraped off large slants into a 1:5000 solution of "Tween 20", and were shaken to bring about more even suspension. For those experiments in which masses of

mycelium were required it was found advisable to use only 1 to 2 week old spore preparations; older spore cultures exhibited a slower growth rate, probably as the result of reduced germination.

Of a number of media used, Difco peptonized milk (PM) appeared to give the most rapid and profuse growth. Cultures were incubated at 25 to 30 C on a reciprocating shaker (100 cycles per minute, 2 inch travel).

RESPIRATION OF MYCELIAL SUSPENSIONS

General Techniques

Respiration was measured by conventional manometric techniques (101) with a bath temperature of 30 C and a shaking rate of 110 strokes per minute with air as the gas phase. Carbon dioxide was absorbed by 0.2 ml. of 20 percent KOH added to the centre well. Mycelium was harvested by centrifugation, and washed with saline or KCl solution (0.02 percent), blended for approximately two minutes in a Potter-Elvejhem homogenizer and suspended in buffer solution. Unless otherwise noted 0.025M phosphate buffer (pH 5.6) was used (28). Cell suspension was added to the main compartment, and substrate (or buffer) was added to the side arm. The flasks were allowed to equilibrate 15 minutes before the stopcocks were closed and measurements begun.

Nitrogen contents of homogenates were determined by Kjeldahl analyses with the digestion mixture of Campbell and Hanna (18). Ammonia was distilled from a Markham distillation unit into 4 percent boric acid, and this was titrated with standard H_2SO_4 to the end-point of a methyl-red bromocresol-green indicator (22).

Preliminary observations indicated that wide variations existed when respiratory measurements were made in the presence or absence of oxidizable substrate. A number of factors were then investigated in order to define those conditions necessary for the preparation of suspensions of assured respiratory activity.

Age of Culture and Amount of Spore Inoculum

Two series of flasks were inoculated with a heavy spore suspension of strain P_{29}. One set received 6.0 ml. of inoculum; the second received 0.5 ml. The cultures were incubated for 2, 3, and 4 days, the mycelium was harvested, and the respiratory activity was measured in the presence of buffer and on the addition of glucose or succinate (5μM). Oxygen uptakes per mgm. of N are shown in FIGURES 1, 2, and 3.

In agreement with the work of other investigators who used a variety of microbial tissues (31, 52, 54), young mycelium appears to be more active than mature cells. This generalization will appear again in the section on organic acid oxidation. With the small inoculum 2-day cultures, there is little difference between exogenous and endogenous O_2 uptake. From such data no conclusions could be drawn regarding the ability of the organism to oxidize these substrates. Older preparations do not exhibit this behaviour to the same degree.

An additional experiment was carried out with similarly established cultures 24 hours old. In this case, the homogenates were allowed to equilibrate for 45 minutes in the presence of substrate before the manometers were closed, and the respiration was recorded. Details of this experiment are presented in FIGURE 4. Again it is observed that the use of mycelium established with large inocula allows for the demonstration of exogenous O_2 uptake which could not be observed when small spore inocula were used. The endogenous

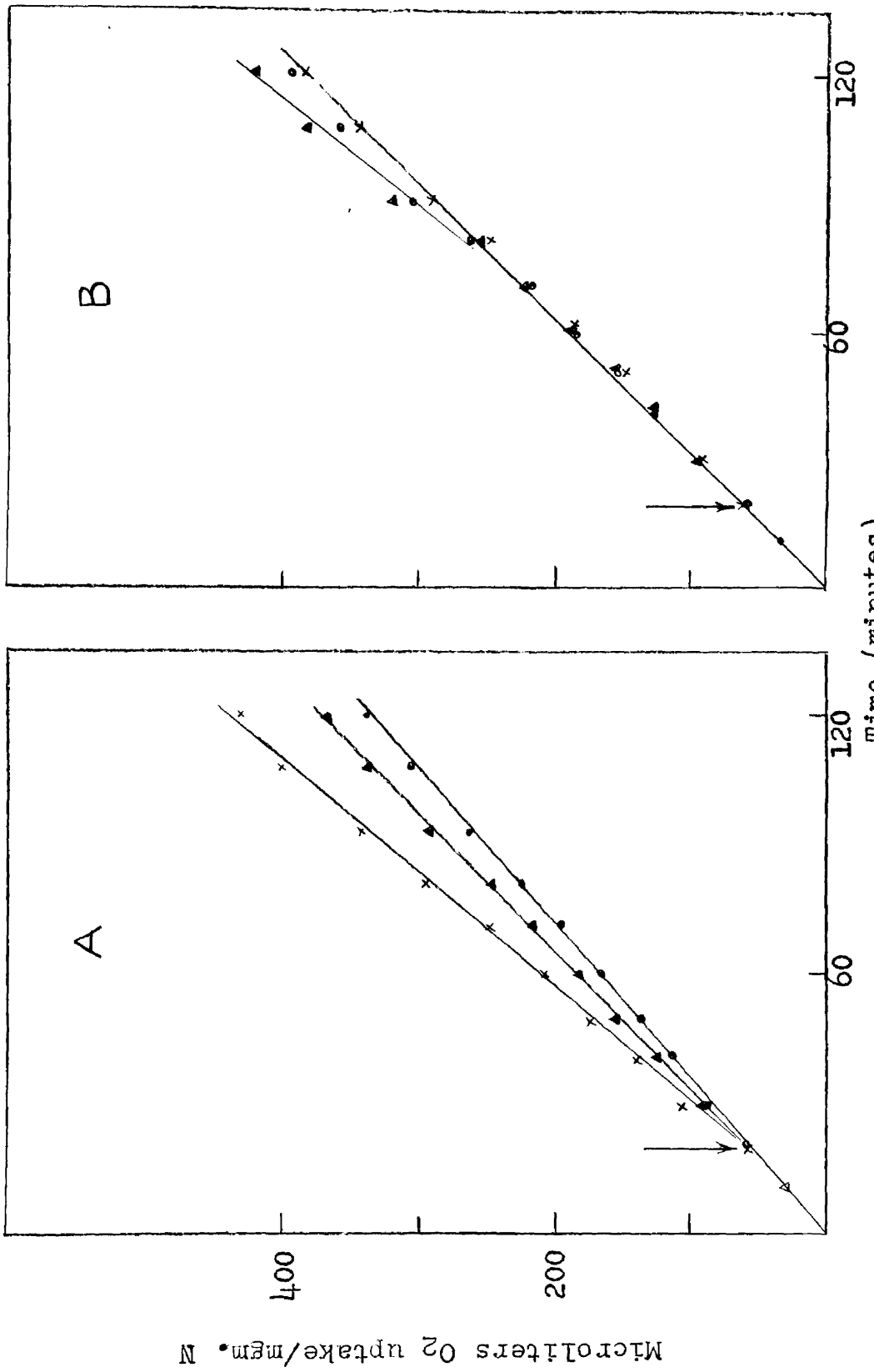

Fig. 1 Oxygen uptake by 2-day mycelial homogenates. A-6.0 ml. inoculum (0.49 mgm. N/flask), B-0.5 ml. inoculum (0.45 mgm. N/flask). Endogenous●—●, succinate, 5μM ▲—▲, glucose, 5μM ×—×. Time of addition of substrate indicated by arrow.

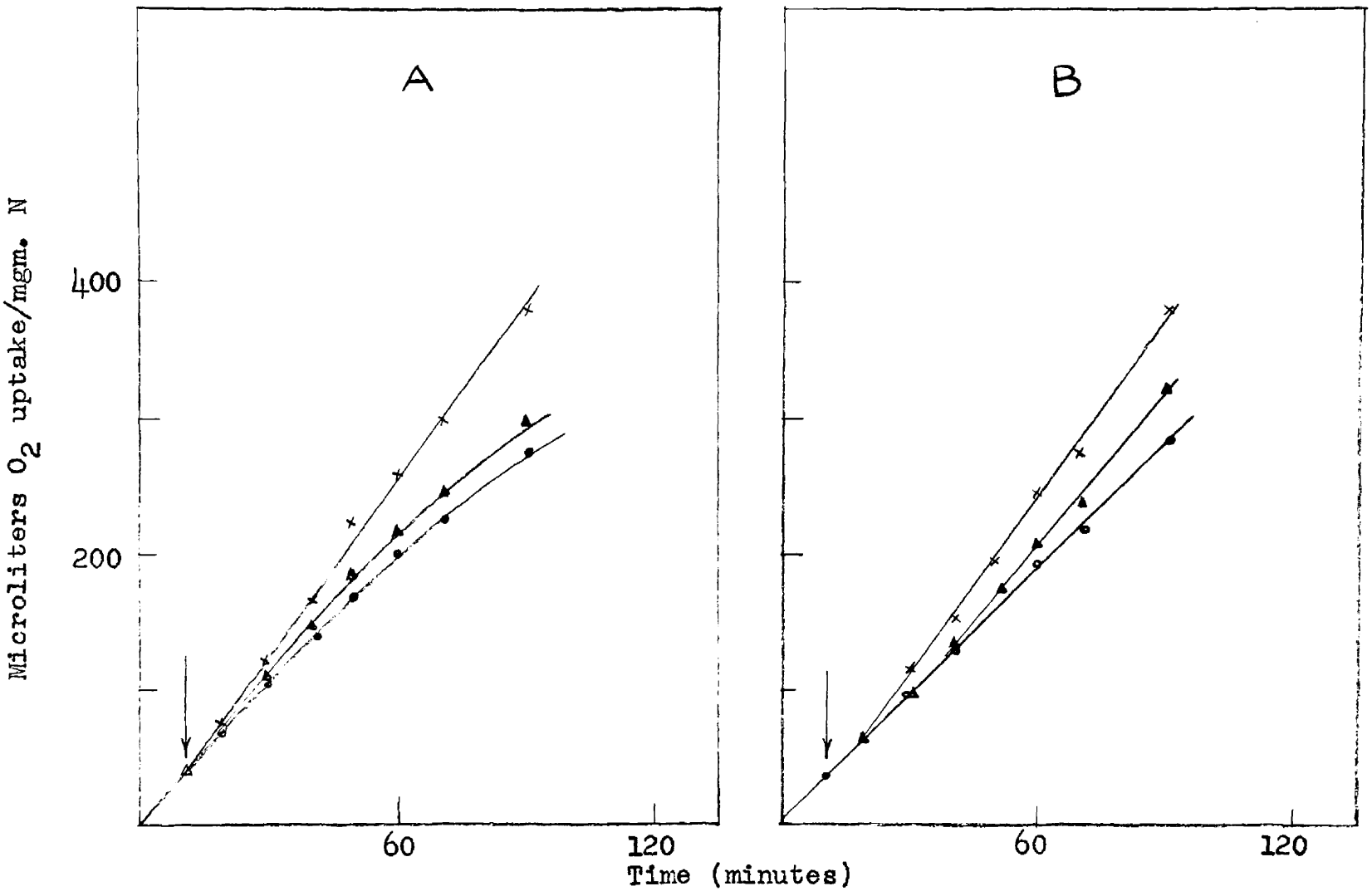

Fig. 2 Oxygen uptake by 3-day mycelial homogenates. A-6.0 ml. inoculum (0.28 mgm. N/flask), B-0.5 ml. inoculum (0.37 mgm. N/flask). Endogenous ●—●, succinate 5μM ▲—▲, glucose 5μM ×—×.

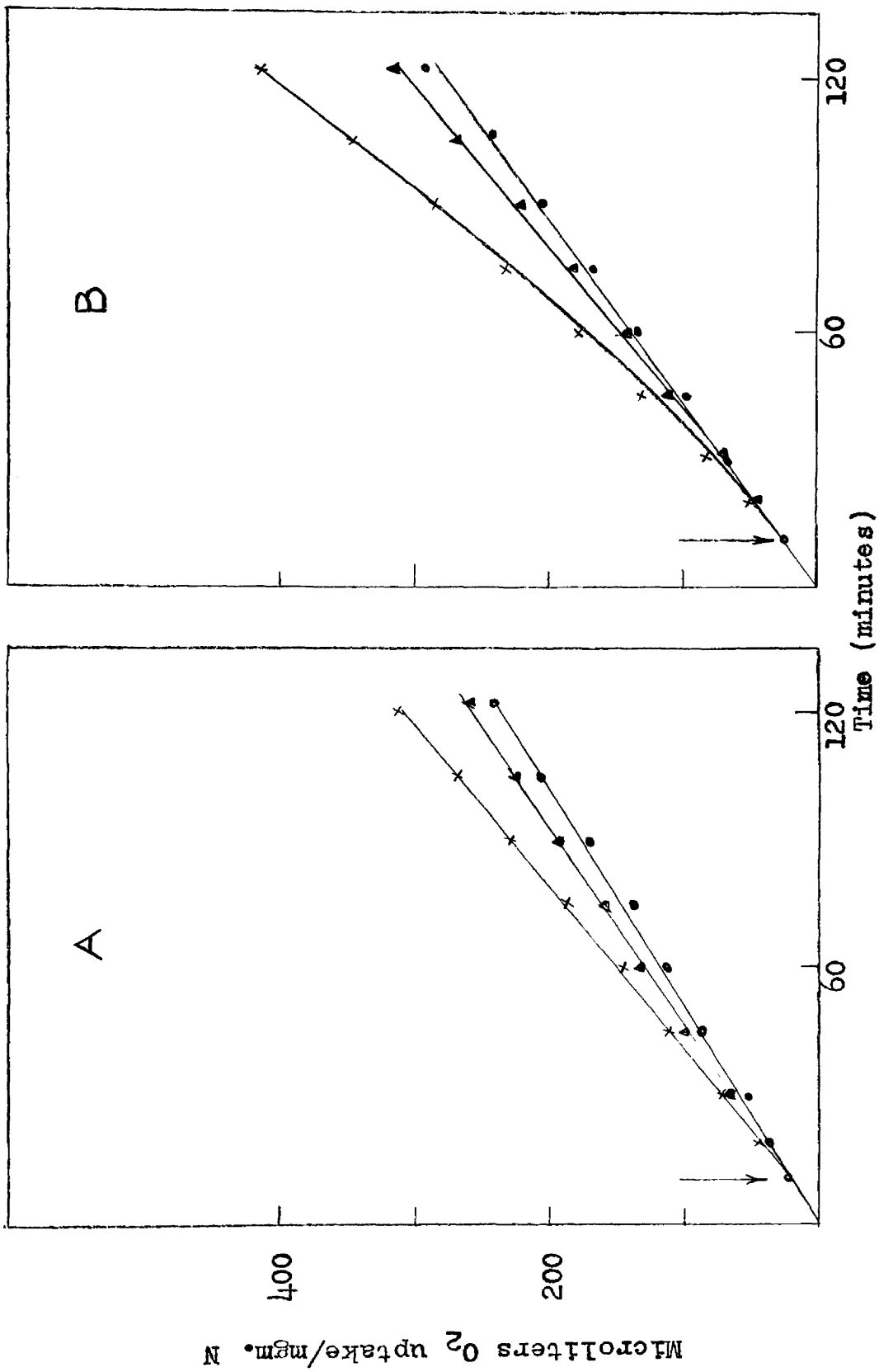

Fig. 3 Oxygen uptake by 4-day mycelial homogenates. A-6.0 ml. inoculum (0.24 mgm. N/flask), B-0.5 ml. inoculum (0.35 mgm. N/flask). Endogenous •—•, succinate 5μM ▲—▲, glucose 5μM ×—×.

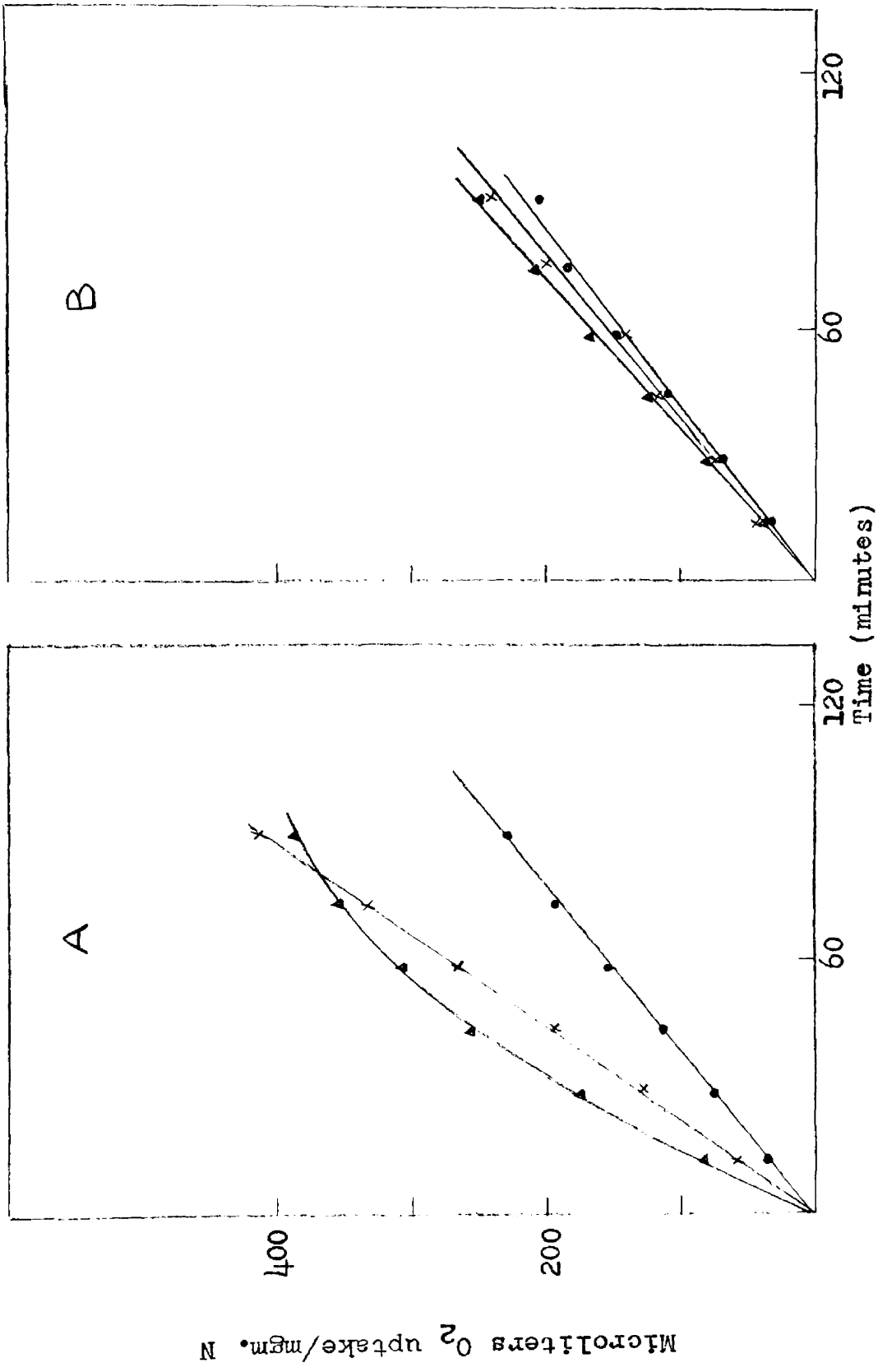

Fig. 4 Oxygen uptake by 24-hour mycelial homogenates. A-6.0 ml. inoculum (0.44 mgm. N/flask), B-0.5 ml. inoculum (0.29 mgm. N/flask). Endogenous •——•, succinate 5μM ▲——▲, glucose 5μM ×——×. Substrates added 45 minutes before readings started.

uptakes are approximately the same in both cases whereas the exogenous uptakes are decidedly different.

Data obtained as described above are clear-cut but misleading, in that the lag which occurs (especially with succinate) before maximum respiratory activity is attained is not evident.

Addition of Glucose to the Growth Medium

The decision to use young mycelium presented difficulties in that adequate yields were more difficult to obtain with short incubation periods. Nickerson and Mohan (75) showed that the addition of glucose increased the growth of S. fradiae in a glutamate medium.

To determine the effect of glucose on cell growth and the time of maximum mycelial development, two sets of flasks were prepared, one containing only PM, the second containing PM plus glucose (PMG) added at the rate of 1.0 ml. of 5 percent solution per 30 ml. of medium. These were inoculated with 1.0 ml. of a filtered spore suspension (P_{29}), and were incubated on the shaker. Duplicate cultures were removed, and the following determinations were made:

Dry weight. Cultures were filtered through tared papers and the mycelium rinsed with 20 ml. of saline. Papers and mycelium were dried overnight at 105 C and re-weighed. Additional filter discs were dried each day to correct for the weight loss on drying.

Total carbohydrate. Filtrates were analysed for total

carbohydrate using the anthrone reagent (73). Preliminary analyses made with this reagent revealed that optical density determinations at 540 mμ would successfully measure an internal glucose standard added to PM.

pH. Hydrogen ion concentration was determined by means of the glass electrode.

Results of these determinations are presented in TABLE 1. It is evident that mycelial growth was maximum at the 2-day sampling, and that the addition of glucose considerably enhanced mycelial yields. No drastic lysis occurred over the interval studied although lysis does occur in older cultures. Carbohydrate disappearance approached the maximum value at 3 days and cultures supplied with additional glucose appeared to cause a very rapid disappearance of the sugar. The reaction became progressively more alkaline during the incubation period presumably because of the liberation of NH_3 (72).

It should be noted that the filtered spore suspension used as inoculum in this experiment did not contain as many spores as the massive inocula used to establish cultures for the respiration trials. With larger inocula maximum yields were obtained more quickly and some lysis was evident at 4 to 5 days. Further additions of glucose did not appreciably increase the growth obtained.

FIGURE 5 shows the respiratory activity of mycelium harvested from PM medium with and without added glucose. The incorporation of glucose into the culture medium appears to

TABLE 1

MYCELIAL WEIGHT, RESIDUAL CARBOHYDRATE, AND pH DURING ACTINOMYCETE GROWTH

Medium	Time days	Dry weight mgms. *	Carbohydrate **	pH
PM	0	-	100	6.7
	1	-	100	7.0
	2	93.5	92.3	8.5
	3	100	81	8.7
	4	94	77	8.7
	5	92	75.5	8.85
PMG	0	-	100	6.7
	1	-	92.5	6.9
	2	171	53	8.0
	3	147	44	8.5
	4	145	44	8.5
	5	145	37.5	8.75

* mycelial weights not determined on the first day - very little growth with the small inoculum.

** carbohydrate expressed as percentage of that in a sample of the uninoculated medium analysed each day.

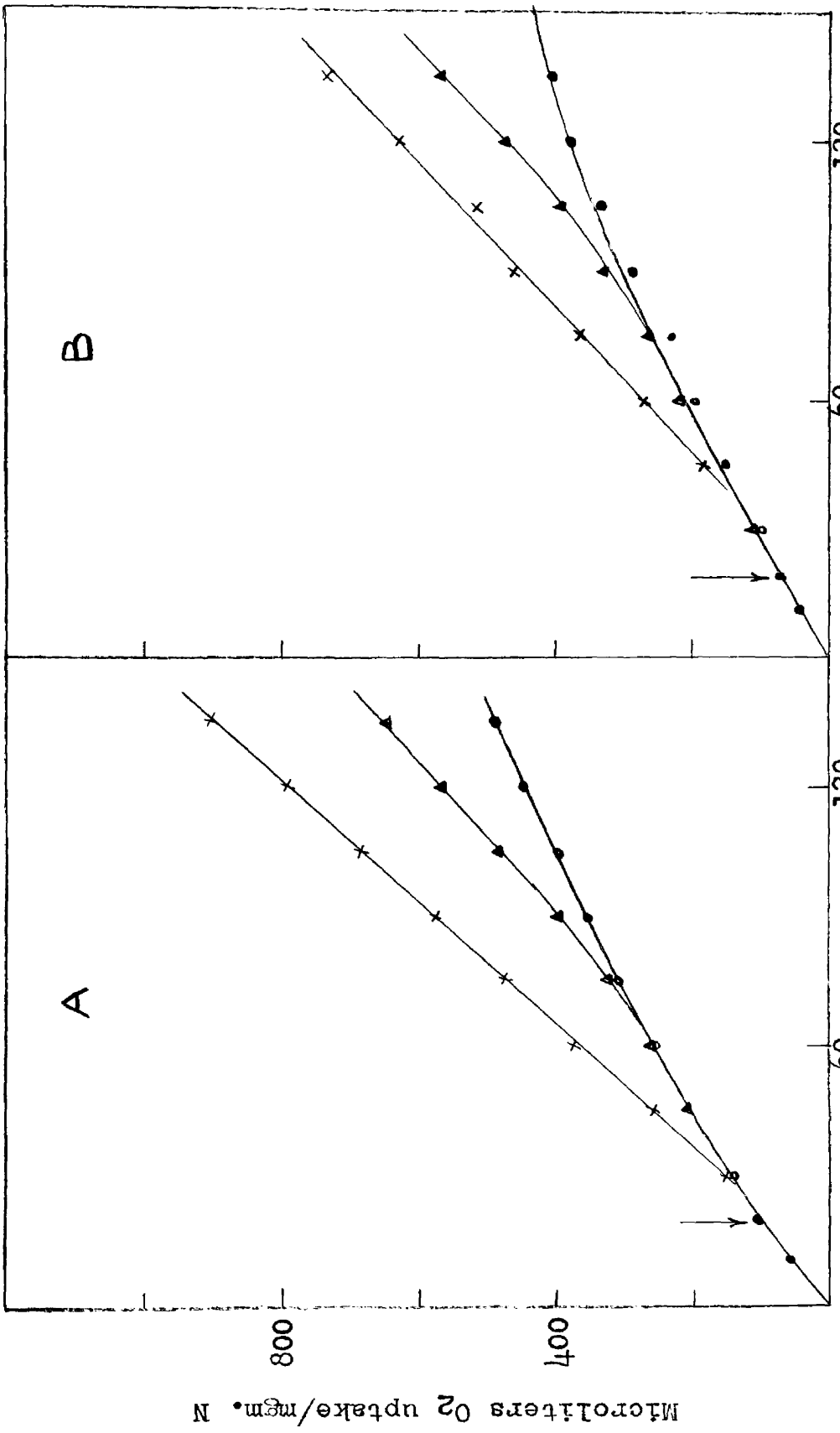

Fig. 5 Oxygen uptake by 16-hour mycelial homogenates grown in PMG (A) or PM (B) medium. Endogenous •——•, succinate 5μM ▲——▲, glucose 5μM ×——×.

stimulate respiratory activity as well as increasing growth.

Adaptation vs Permeability Effects

Data in FIGURE 5 indicated that a lag occurred in the attainment of maximum respiratory rate when succinate was added to homogenates, and suggested that this type of response might be inductive in nature. To test this possibility mycelium was grown in PMG and in PMG plus succinate (1.0 ml. 5 percent sodium succinate per 30 ml. medium); the cellular material was harvested and the respiration observed with glucose or succinate as the substrate. Results of these experiments are given in FIGURE 6.

The incorporation of succinate into the growth medium appears to eliminate the lag encountered before the maximum rate of succinate oxidation begins. This response is observed consistently and is more evident when succinate is added to the Warburg vessels in low concentration (5μM per flask). With PMG cells the maximum respiration rate is achieved more rapidly when the succinate concentration is increased (25μM per flask). This reduction in lag by increasing concentration is shown in TABLE 2.

These data indicate that the presence of succinate in the medium in some way "conditioned" the cells so that the effect of increasing the substrate concentration is no longer apparent.

On the surface this appears to be a clear demonstration of an inductive response; however, permeability effects have

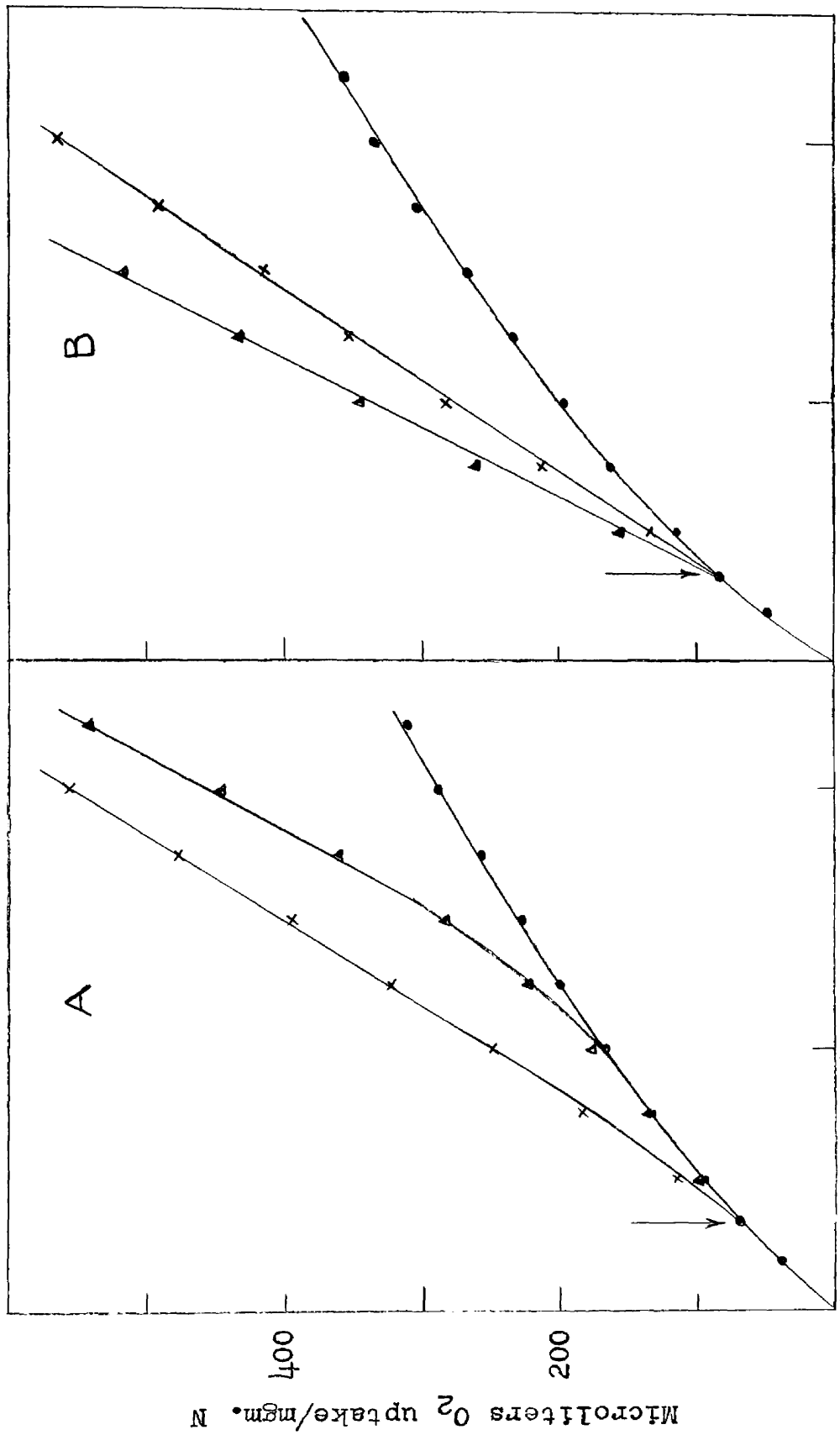

Fig. 6 Oxygen uptake by 15.5-hour mycelial homogenates grown in PMG (A) or PMG succinate (B). Endogenous ●——●, succinate 5μM ▲——▲, glucose 5μM ×——×.

been shown to cause similar results with Azotobacter (82). Definitive resolution of this point could be achieved by showing an immediate activity on succinate in mycelial extracts (see section on cell extracts).

TABLE 2

EFFECT OF THE ADDITION OF SUCCINATE TO THE MEDIUM ON THE OXIDATION OF SUCCINATE BY MYCELIAL HOMOGENATES

Concentration of succinate μM/flask	Respiration* for period 20-120 minutes for mycelium grown in			
	PMG		PMG + succinate	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
5	135	187	208	183
25	226	250	200	176

* expressed as percentage of endogenous.

Oxidation of Organic Acids

The respiration of young and old mycelial homogenates in the presence of several metabolically important organic acids is shown in TABLE 3. These data indicate that cell suspensions can oxidize succinate, fumarate, citrate, and acetate. Succinate and fumarate in particular are oxidized very vigorously. It is interesting to note that the presence

TABLE 3

RESPIRATION OF YOUNG AND OLD MYCELIAL HOMOGENATES

17-hour homogenate

Substrate	Respiration for period *			
	0-60	60-120	120-180	0-180
endogenous	383	195	182	760
glucose	480	394	382	1256
succinate	458	520	805	1783
fumarate	408	682	484	1574
citrate	451	415	238	1104
acetate	495	374	318	1187

96-hour homogenate

Substrate	Respiration for period *			
	0-60	60-120	120-180	0-180
endogenous	142	122	99	363
glucose	225	243	312	780
succinate	171	259	274	704
fumarate	180	232	301	713
citrate	117	130	139	386
acetate	195	227	171	593

* μl. O_2 uptake/mgm. N. Time periods are given in minutes.

of citrate appears to exert a depressing effect on 4-day cells; in addition, there is a lag of approximately 2 hours before the respiratory rate begins to exceed that of endogenous controls.

Also evident from the above data is the fact that young mycelium is more active than older mycelium, and therefore appears to be the preferred material for respiratory investigations. This observation might have been anticipated from growth data previously given. These data indicated that net cellular synthesis had ceased after 2 days incubation. It should be pointed out, however, that the growth data was obtained using a filtered spore suspension as inoculum whereas the mycelium for respiration was derived by using massive spore inocula. Larger inocula cause the growth rate to be increased, and net synthesis is probably completed at an earlier time in the incubation period.

Effect of Starvation of the Mycelium

In all these experiments the high and persistent endogenous respiratory activity of homogenates is evident. An experiment was carried out in which a cell suspension was "starved" in buffer for 3 hours on the shaker before it was used in the Warburg vessels. The respiratory activity of this preparation and of a sample of the same suspension not subjected to this preliminary starvation is given in FIGURE 7.

First observation would indicate that the shaking procedure has seriously interfered with the respiratory activity of the homogenate. Closer examination of the data, however,

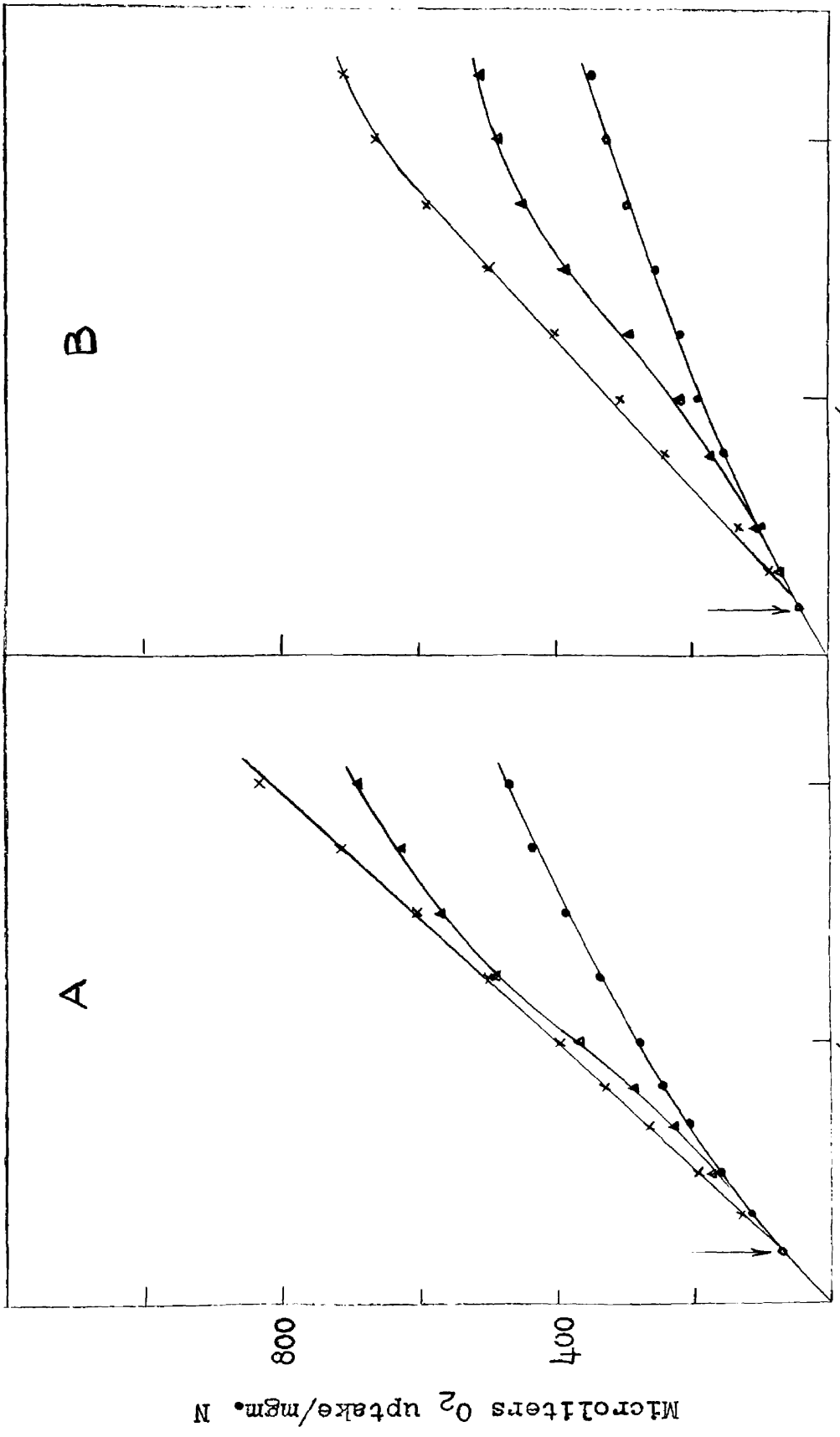

Fig. 7 Oxygen uptake by 16-hour mycelial homogenate. A-not starved, B-starved on shaker for 3 hours. Endogenous ●——●, succinate 5μM ▲——▲, glucose 5μM ×——×.

shows that the initial respiratory activity of the non-starved mycelium is, for the most part, responsible for the difference in activity noted. The $Q_{O_2}(N)$ values calculated from the slopes of the O_2 uptake curves show that for non-starved mycelium the Q_{O_2}, which is initially 375, falls to about 200 during the second hour of respiration. For starved cells the initial value is 200, and during the second hour decreases to 173 - a decrease of only 12.5 percent compared with a 46.7 percent decrease for the unstarved suspension. These data show that after approximately 1 hour the rate of endogenous respiration decreases, and following this remains relatively constant for 4 to 5 hours (that is, 3 hours starvation plus 2 hours in the respirometer).

Data from a number of experiments are collected in TABLE 4 to show that this rapid initial respiration and subsequent decline are consistently observed. The trend is especially noticeable in young cultures. An experiment was carried out in which the endogenous and exogenous respiration were followed for about 3 hours; at approximately 1 hour intervals the manometers were opened, reset, closed, and respiration again measured. The results of this experiment are shown in FIGURE 8. It is evident that endogenous activity decreases significantly after 1 hour, after which the slope of the line is relatively constant over the time period observed. Exogenous respiration does not exhibit this significant rate decrease.

These data indicate that a short (1 to 1.5 hour)

starvation period significantly reduced the endogenous respiration rate, and in much of the subsequent work this technique was used in order to lower the control uptake values.

TABLE 4

THE DECREASE IN ENDOGENOUS RESPIRATION OF MYCELIAL HOMOGENATES

Mycelial age (hours)	Respiration for the period			
	0-30	30-60	60-90	90-120
15.5	100	65	62	61
16	109	105	85	83
17	177	140	118	55
22	114	100	76	--
72	102	100	85	--
96	77	65	66	57

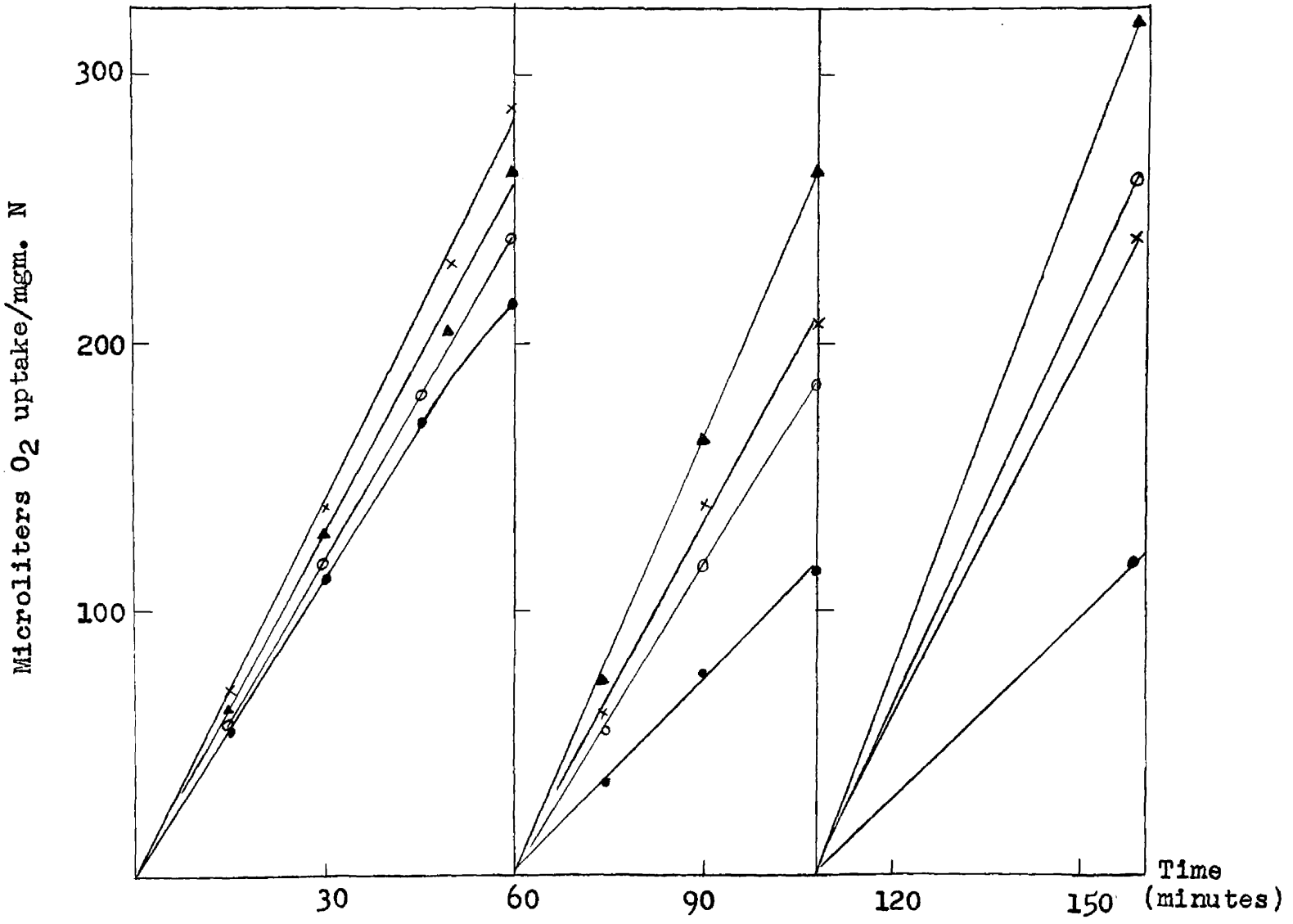

Fig. 8 Oxygen uptake of 22-hour mycelial homogenate over 3 time periods. Endogenous ●——●, succinate ▲——▲, citrate ○——○, glucose ×——×. Substrates at 12.5 μM/flask.

The Oxidation of Amino Acids

Experiments were carried out to determine the ability of cell suspensions to oxidize a number of amino acids. Mycelium harvested by centrifugation from PMG medium was washed in saline, disrupted, suspended in phosphate buffer (pH 5.6, 0.025 M), and starved on the shaker for 1 hour. Amino acid substrates were neutralized with NaOH and added to Warburg vessels at level of 50μM per flask. The amino acids used were L forms except for valine, phenylalanine, and threonine which were racemic mixtures. FIGURE 9 shows the results of these trials.

Following respiration, the flasks were removed, the reaction stopped by the addition of 0.5 ml. of 20 percent trichloroacetic acid (TCA) solution, the contents of duplicate flasks pooled, and the precipitated mycelium removed by centrifugation. Samples of the supernatant fluid were analysed for NH_3 by direct nesslerization. The results of these analyses along with total O_2 uptakes for the 3 hour respiration period (uptake before the addition of substrate has been subtracted) are collected in TABLE 5.

The data show that leucine, valine, glycine, and phenylalanine are but slowly oxidized over the 3 hour period studied whereas the other amino acids, particularly glutamic and aspartic, are oxidized very vigorously. Thus, for cells grown in a casein digest the amino acids appear to represent a considerable potential energy source for the organism. The intense oxidation of aspartic and glutamic acids, coupled

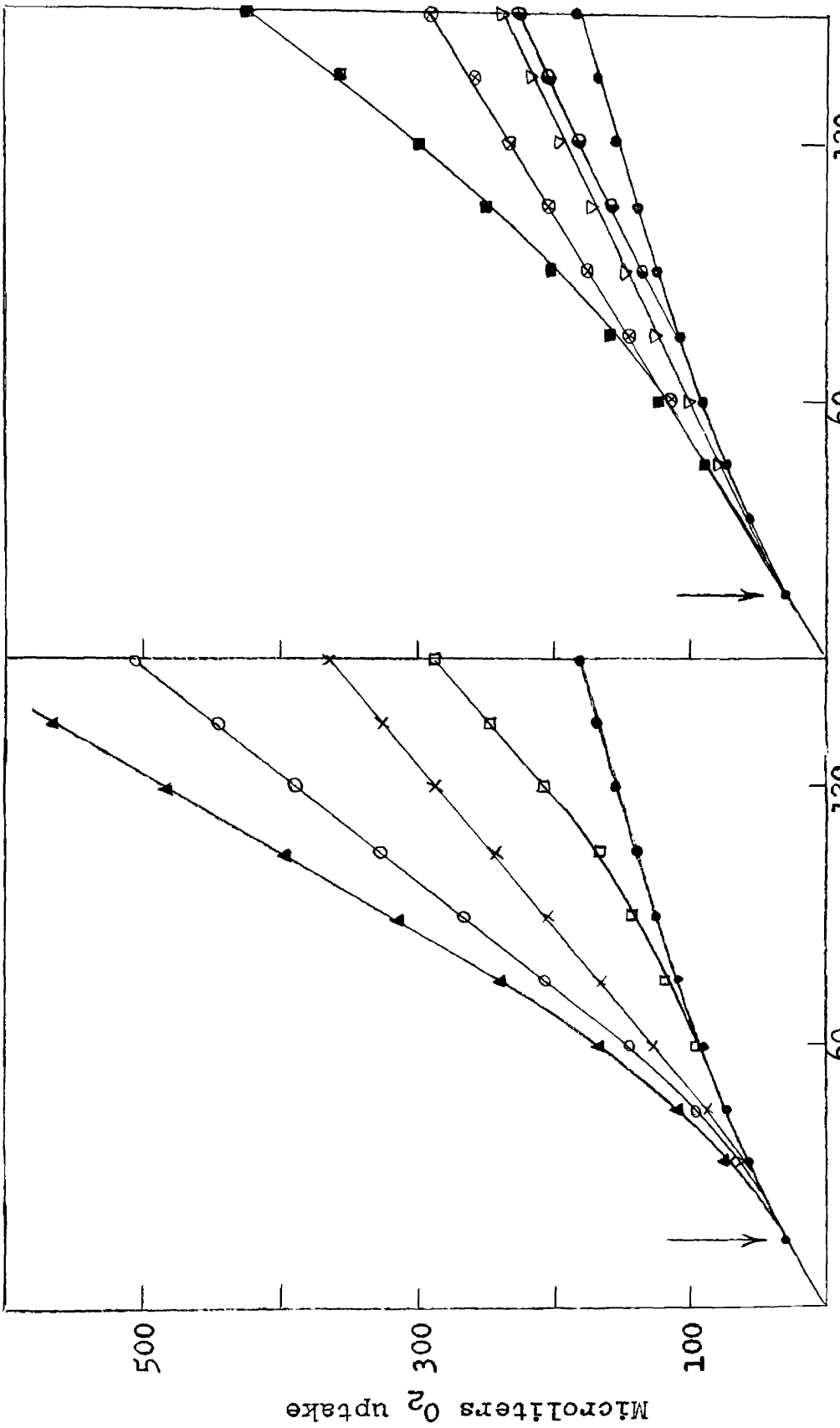

Fig. 9 Oxidation of amino acids by 15.5-hour mycelial homogenate (starved 1 hour). Endogenous ●—●, glutamate ▲—▲, aspartate ○—○, threonine ×—×, phenylalanine □—□, alanine ■—■, glycine ⊗—⊗, valine ▽—▽, leucine ⊙—⊙. Phenylalanine added as a saturated solution; others at 50μM/flask.

TABLE 5

OXYGEN UPTAKE AND AMMONIA RECOVERY DURING THE OXIDATION OF AMINO ACIDS

Substrate	Oxygen uptake *	Exogenous / Endogenous **	μM NH_3 recovered
endogenous	175	100	7.2
glutamate	820	468	14.4
aspartate	579	330	15.8
threonine	427	244	13.2
phenylalanine	349	199	5.0
valine	251	143	6.6
alanine	540	308	14.4
leucine	250	143	5.9
glycine	321	183	12.8

* μl. O_2 consumed for period 15-180 minutes.

** expressed as percentage of endogenous control.

with their activity in transaminating systems, emphasizes the pivotal role of these compounds in amino acid and energy metabolism.

The yields of NH_3 are far below what would be expected if simple oxidative deamination were the only reaction to take place, viz:

$$R - \underset{\displaystyle NH_2}{\overset{\displaystyle H}{\overset{|}{\underset{|}{C}}}} - COOH + 0.5\ O_2 \longrightarrow R - \underset{\displaystyle O}{\overset{\displaystyle O}{\overset{||}{C}}} - COOH + NH_3$$

In the above case an O_2 consumption of 11.2 μl. would be expected to occur for each μM of NH_3 formed. The actual O_2 uptakes far exceeded this figure, so that it must be assumed that the residual carbon skeleton of the amino acid is amenable to further oxidation. Even leucine and valine support a greater O_2 uptake than can be accounted for by simple deamination to the corresponding keto acid.

FIGURE 9 also shows that a significant lag occurs prior to the maximum activity on alanine and phenylalanine. Following this lag, the rates are fairly rapid. The slow oxidation of valine and leucine is interesting in view of the similarity in structure of these acids.

Endogenous Respiration and the Respiratory Quotient

The characteristic endogenous respiratory activity of mycelial homogenates and the steps taken to reduce it have been discussed (see pages 32 and 33). The nature of the

endogenous substrate is not known, and attempts to detect a loss of carbohydrate after respiration by staining methods (91) were not successful. In an effort to determine the source of this endogenous activity the respiratory quotient (R.Q.) was determined both from endogenous reserves and upon the addition of succinate, pyruvate, or glutamate. In the simplest case the R.Q. would be expected to be unaltered by the addition of substrate similar in nature to that already being oxidized. Oxygen uptake was measured as previously described, and CO_2 evolution was measured by Warburg's "direct method" with a correction being made for CO_2 retention in the buffer (101).

FIGURES 10 and 11 show data obtained for oxygen consumption and CO_2 evolution. The time lags which occurred before maximum activity are again evident especially in the case of pyruvate. For this substrate the oxidation appears to be composed of two distinct phases, a situation which has previously been reported by Barrett et al (6) for the oxidation of citrate by a Pseudomonas species. These workers suggested that an actual adaptation was occurring during which process an induced synthesis of "carrier molecules" which were concerned with substrate transport across the cell membrane was brought about.

TABLE 6 indicates R.Q. values tabulated over three time intervals during the course of respiration. Values have also been calculated from the slopes of the curves at points of maximum activity. All three compounds caused a large initial

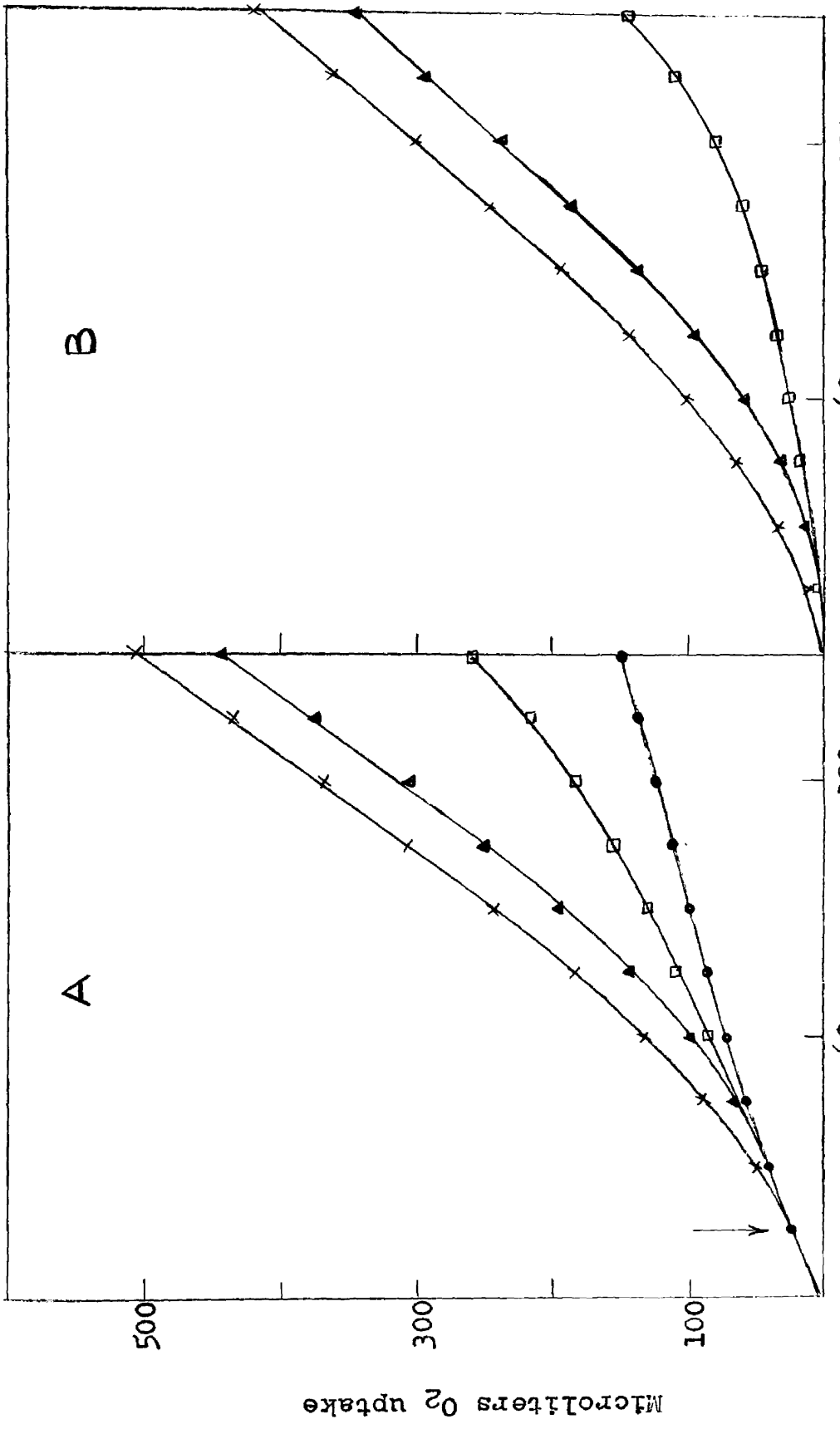

Fig. 10 Oxygen uptake by 15.5-hour homogenate (0.43 mgm. N/flask). Endogenous ●—●, succinate ▲—▲, glutamate ×—×, pyruvate □—□. Substrates at 25 μM/flask. A-endogenous not subtracted, B-endogenous subtracted.

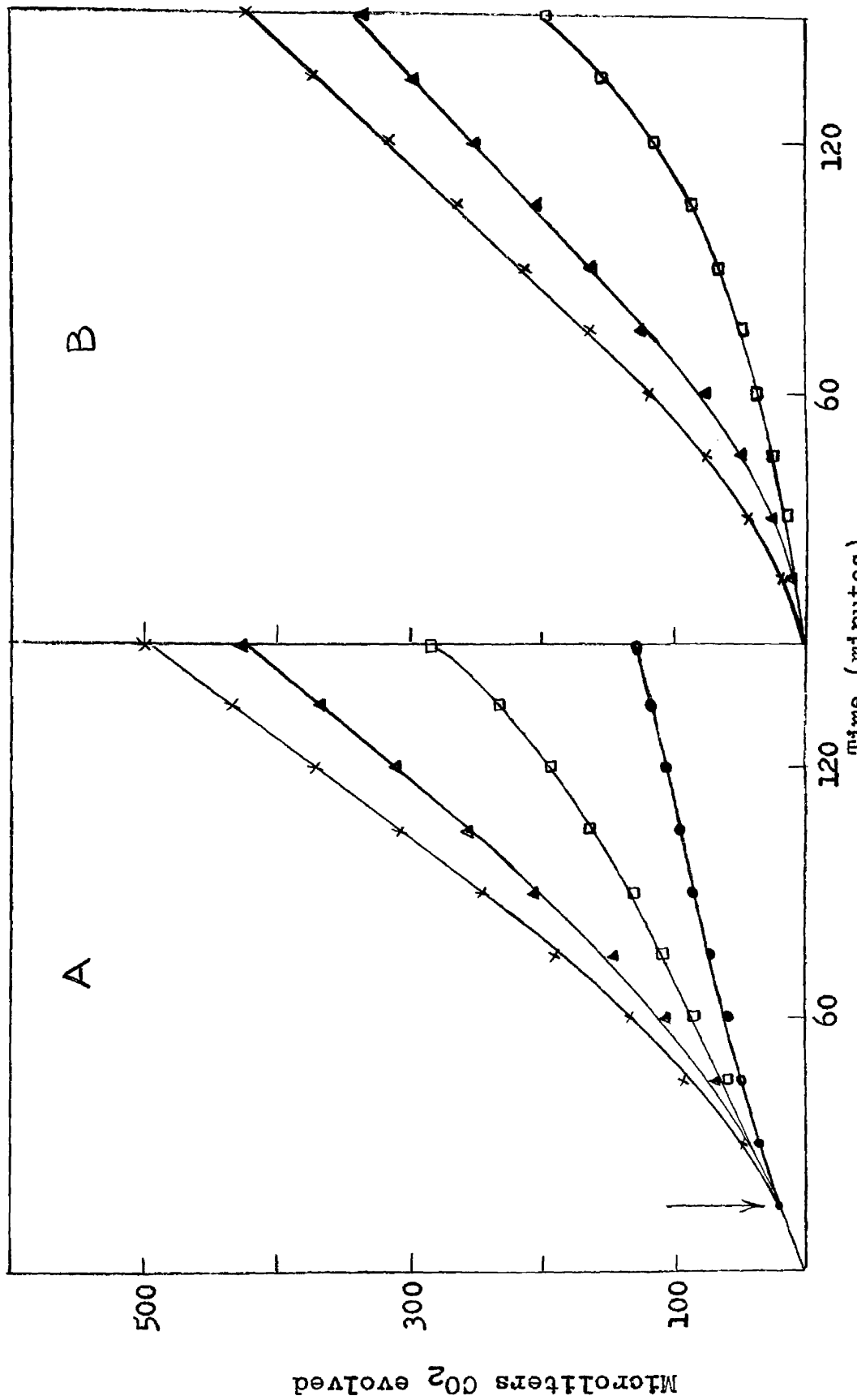

Fig. 11 Carbon dioxide evolution by 15.5-hour homogenate. Endogenous ●——●, succinate ▲——▲, glutamate ×——×, pyruvate □——□. Substrates at 25 μM/flask. A-endogenous not subtracted, B-endogenous subtracted.

TABLE 6

VARIATION IN THE RESPIRATORY QUOTIENT DURING THE OXIDATION OF GLUTAMATE, PYRUVATE, AND SUCCINATE

Substrate	R.Q. over indicated time interval						$\frac{Q_{CO_2}(N)}{Q_{O_2}(N)}$
	Endogenous included			Endogenous subtracted			
	15-60	60-120	120-180	15-60	60-120	120-180	
endogenous	.87	.89	.86	-	-	-	0.86
glutamate	1.01	1.01	0.91	1.11	1.05	0.92	0.94
pyruvate	1.02	1.15	1.22	1.47	1.45	1.34	1.26
succinate	1.17	0.98	0.76	1.59	1.00	0.74	0.88

increase in R.Q. values which decreased as respiration progressed. Data calculated from the slopes of the lines, however, may be more significant in view of the lag periods previously mentioned. The addition of pyruvate caused a significant rise in R.Q., a situation to be expected if decarboxylation were the mode of attack.

The endogenous R.Q. value was relatively constant ($\approx$0.86) which suggested that at least part of the respiration probably arose as the result of protein degradation. Other data obtained in the study of amino acid oxidation indicated that significant quantities of NH_3 were produced, and this observation supported the concept that endogenous activity was, in part at least, carried on at the expense of nitrogenous reserves. Chromatographic analyses of cell extracts indicated that considerable amounts of free amino acids were present (see section on transamination). These substances could serve as substrates for endogenous activity. The respiration of homogenates in buffer solution was accompanied by an increase in non-protein nitrogen (NPN), a condition to be expected if protein contributed to the endogenous activity. A prominent amino acid constituent of cell extracts was glutamic acid, the addition of which elevated the R.Q. obtained. It is suggested that if the endogenous respiration were the result of amino acid oxidation then the significant quantities of glutamic acid which were present may have been sufficient to cause the R.Q. to be in excess of the expected value for protein degradation.

Another point evident from FIGURE 10 is that the respiratory rates on succinate and glutamate are very similar. ($Q_{O_2}(N)$ values are succinate 587, glutamate 609, endogenous not subtracted). A possible explanation is that glutamate oxidation proceeds as follows:

$$\text{glutamate} \xrightarrow{A} \text{succinate} \xrightarrow{B} \text{end products}$$

and that the steps A and B are dissimilar in rate, step A being the more rapid. This condition would result in the all-over rate being determined by B, the rate-limiting step. The Q_{O_2} values would be expected to be the same if the above were true. Further support for this pathway of glutamate degradation is developed in the section on α-ketoglutarate oxidation.

Effect of Dinitrophenol (DNP)

The data previously discussed indicated that the stimulation of respiration caused by the addition of an exogenous oxidizable substrate was not as striking as that obtained with some other microorganisms (76, 91). Also evident was the fact that the extra oxygen taken up in the presence of added substrate was much below that which would be expected for complete oxidation; this fact suggested that assimilation was occurring. Various workers have shown that DNP inhibits assimilation and thus allows for the more complete oxidation of added substrate than would occur in its absence (2, 7, 51). The process of phosphorylation which accompanies

oxidation, and which supplies the energy for the process of assimilation, is thought to be inhibited by this compound (2, 48). With most tissues, low concentrations considerably increase respiration whereas higher concentrations are inhibitory (88). The action of DNP on respiration is strongly pH sensitive as shown by the extensive investigations of Simon (88). Data obtained by this worker are of considerable value in establishing the concentrations of the inhibitor which must be employed. An experiment was carried out to determine whether this compound could be used to increase the difference between the exogenous and endogenous respiration of actinomycete homogenates.

A 10^{-3} M stock solution of 2, 4-dinitrophenol was prepared; and from this, dilutions were made so that the addition of 0.5 ml. of solution established concentrations of $5x10^{-5}$, 10^{-4}, and $2.5x10^{-4}$ M in the Warburg vessels. The effect of these concentrations on endogenous and glucose respiration was then observed.

FIGURE 12 presents the results of one experiment. At a concentration of $5x10^{-5}$ M, DNP stimulated endogenous respiration whereas at $2.5x10^{-4}$ M both exogenous and endogenous respiration were depressed. The results at 10^{-4} M were more difficult to interpret. In five experiments the 10^{-4} M concentration gave an increase in endogenous respiration whereas in another experiment the O_2 uptake was not appreciably greater than that of the control. It is suggested that this concentration represents a critical level at

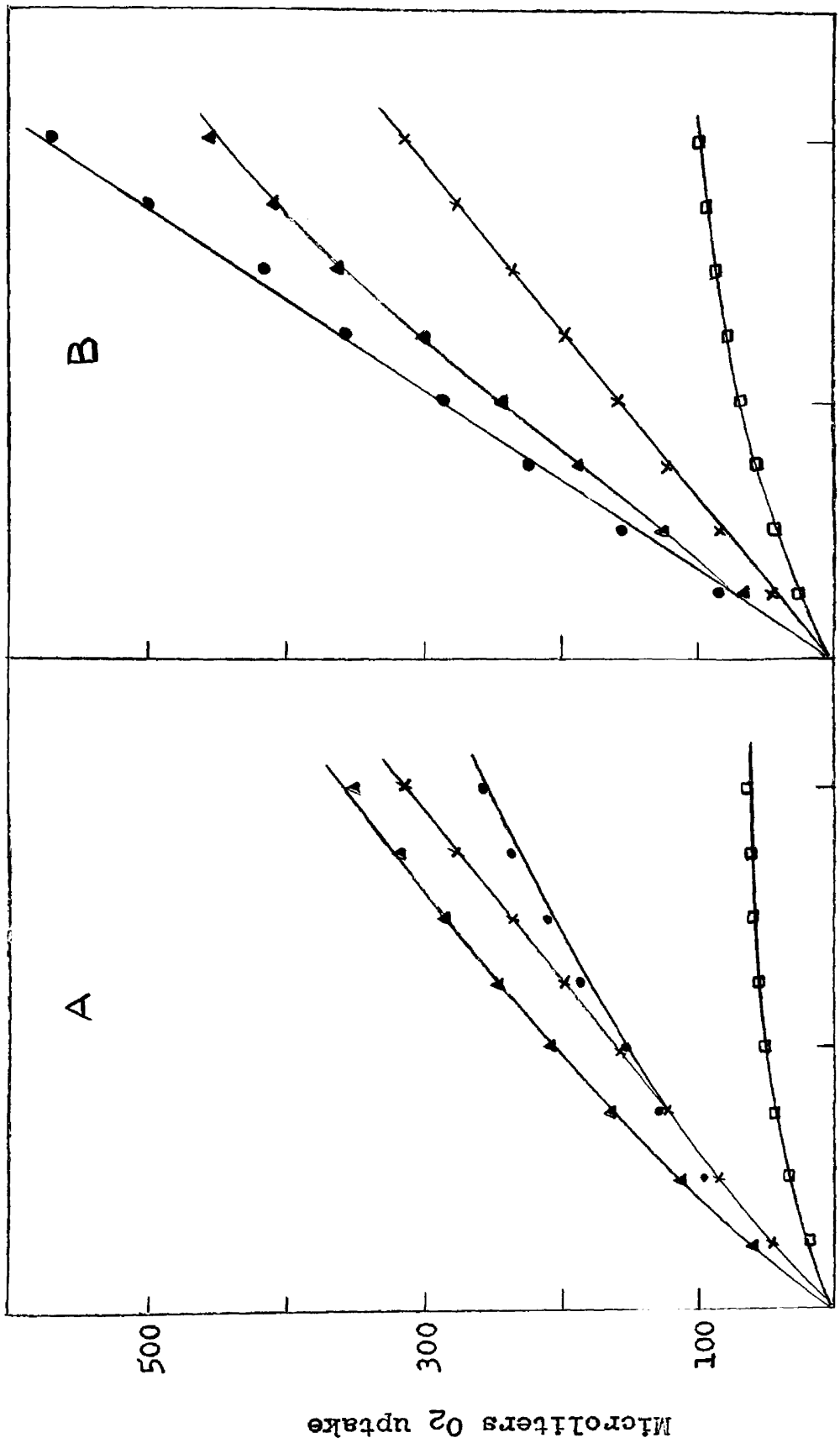

Fig.12 Oxygen uptake by 22-hour mycelial homogenate (0.59 mgm. N/flask). A-endogenous, B-plus 25μM glucose/flask. No DNP ●——●, 5x10-5 M DNP ▲——▲, 1x10-4 M DNP ×——×, 2.5x10-4 M DNP □——□.

which the opposing reactions of stimulation and inhibition are rather delicately balanced.

The oxidation of added glucose appeared to be depressed by all concentrations of DNP tested. At a concentration of 2.5×10^{-4} M this effect was probably the result of the inhibition of phosphorylation, a synthetic process presumably required for glucose oxidation. The lower DNP concentrations, however, were expected to show an increased rate of glucose oxidation over that of the controls.

These apparently anomalous results were clarified when it was found that the pattern of glucose oxidation in the presence of DNP was strikingly changed by preliminary starvation of the mycelial homogenates. The experiments outlined above were repeated with 5×10^{-5} and 10^{-4} concentrations of DNP using cells which had been starved for 1 hour in buffer solution. Results of these experiments are shown in FIGURE 13.

Here again the stimulation of endogenous respiration by DNP is observed but in addition the expected stimulation of glucose oxidation by low DNP concentrations is evident. In these latter experiments the glucose concentration was reduced to 2 μM/flask, an amount which on complete oxidation was expected to yield an O_2 uptake of $\approx 267 \mu$l. By the end of the 2 hour observation period the uptake rates had fallen to the level of those in the flasks containing no glucose; upon subtraction of endogenous from exogenous uptakes it was observed that the addition of DNP had increased the percentage oxidation to 62 percent (5×10^{-5} M) from a value of only

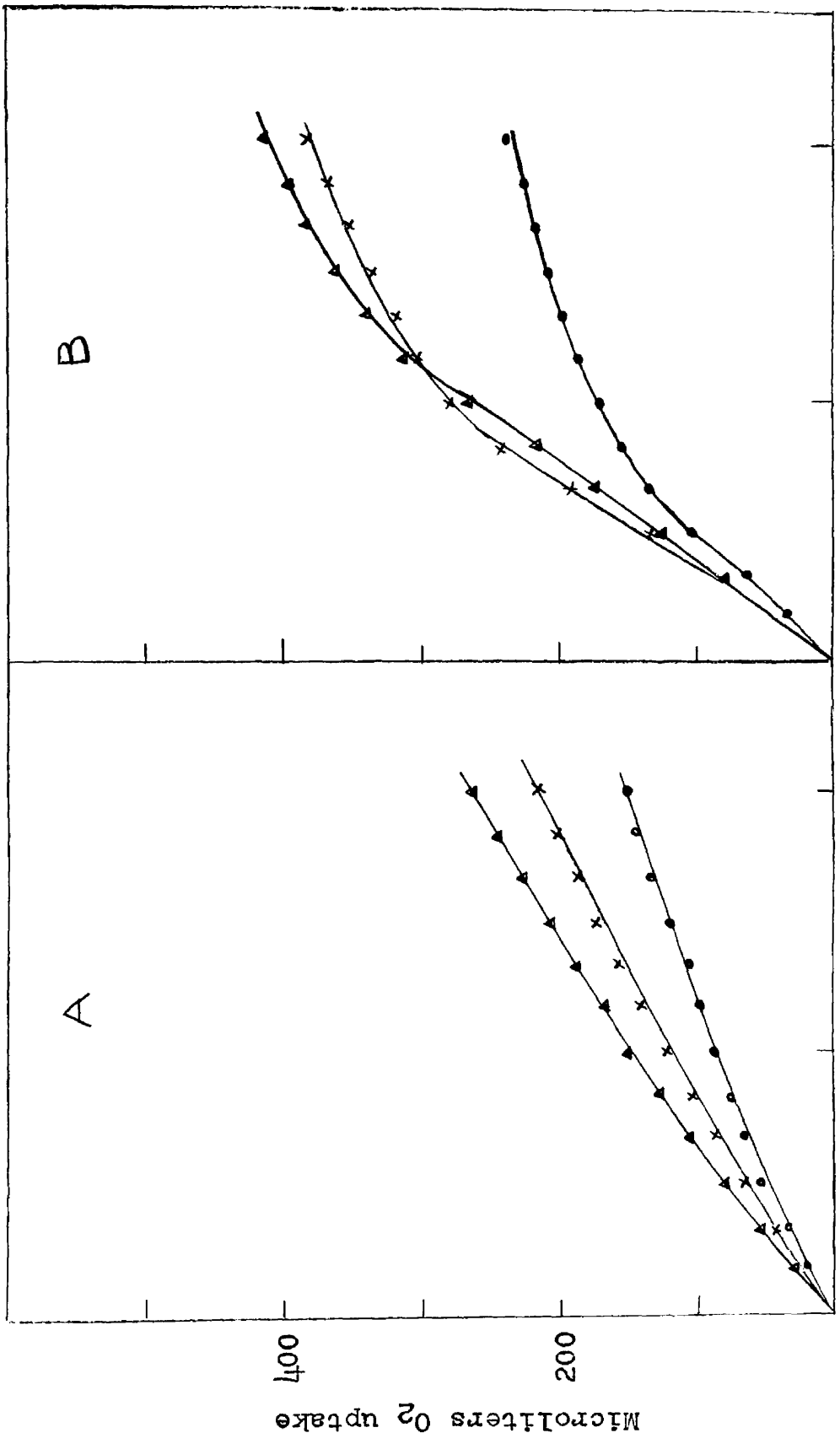

Fig. 13 Oxygen uptake by 17.5 hour mycelial homogenate (0.56 mgm. N/flask). A-endogenous, B-plus 2μM glucose/flask. No DNP ●——●, $5x10^{-5}$ M DNP ×——×, $1x10^{-4}$ M DNP ▲——▲.

33 percent in the absence of DNP.

This stimulation of glucose oxidation by DNP was more striking with older cells, and as mentioned above could be demonstrated only after preliminary starvation. These observations suggest that the DNP effect is in some way dependent upon the level of endogenous reserves within the homogenate. The data are consistent with the concept that when the reserve level is high some naturally present regulatory mechanism controlling the relative amounts of glucose assimulated and oxidized operates in favor of the latter process and prevents the demonstration of the DNP effect. Further work will be necessary to define more precisely the variables involved.

The Oxidation of ∝-ketoglutarate

Considerable difficulty was encountered in attempting to demonstrate the oxidation of ∝-ketoglutarate by cell homogenates. In most cases, the presence of this compound caused no significant change in respiration over that of the control. Since this compound is a key intermediate in the Kreb's cycle and also plays a pivotal role in amino acid metabolism it was thought important to demonstrate its utilization directly or indirectly.

Cell homogenates were able to bring about the oxidation of another keto acid, pyruvic acid. In this oxidation the usual lag was noted but following this a definite stimulation of respiratory activity occurred. Terner (93, 94),

working with homogenates of mammary gland tissue, showed that DNP depressed the endogenous respiratory activity of his preparations and allowed for the demonstration of an exogenous respiration which otherwise could not be detected. The data of Terner indicated that the action of DNP was upon the oxidation of keto acids or their direct precursors.

The effect of DNP on the oxidation of pyruvate and α-ketoglutarate was examined. Cell homogenates were combined with substrate (50μM/flask) and DNP, and allowed to equilibrate for 20 minutes in the Warburg vessels. The manometers were then closed, and O_2 uptakes recorded.

Results of this experiment are shown in TABLE 7.

TABLE 7

EFFECT OF DINITROPHENOL ON
KETO ACID OXIDATION

Substrate	Respiration with DNP concentration of		
	0	10^{-4} M	$5x10^{-5}$ M
endogenous	302	398	328
α-ketoglutarate	303	397	349
pyruvate	418	448	429

μl. O_2 uptake by a homogenate from an 18-hour culture of P_3. Observation period 150 minutes. Homogenate not starved so endogenous value is high. 1.0 ml. of homogenate (0.54 mgm. N/ml.) per flask; total volume 2.2 ml.

The data show that DNP affected both endogenous and exogenous respiration to about the same degree; certainly no significant stimulation of keto acid oxidation occurred because of the presence of DNP. The ratio of exogenous/endogenous oxygen uptake with pyruvate was slightly depressed by the presence of DNP at both concentrations tested. Higher concentrations were not used since it was found that 2.5×10^{-4} M DNP significantly reduced both endogenous activity and also exogenous activity with added glucose. Further work on the effect of DNP was not done because it was found that α-ketoglutarate oxidation could be demonstrated directly.

During the observations on amino acid oxidation it was noted that oxygen uptakes were significantly higher than would be expected for simple deamination (as calculated from NH_3 recoveries). With glutamate as the substrate the O_2 uptake was considerably greater than calculated for glutamic dehydrogenation, and the rate of respiration closely approximated that of added succinate. Such a condition would occur if glutamate were deaminated to α-ketoglutarate, the latter oxidized to "succinate" and this in turn further degraded (provided the previously suggested condition with respect to rates prevailed). If glutamate is oxidized through succinate then an inhibitor of succinate oxidation should also yield an inhibition of glutamate oxidation provided the sequence of events suggested above actually takes place.

The classical competitive inhibition of succinic dehydrogenase by malonate first discovered by Quastel and co-workers

has been repeatedly confirmed (62, 96). To test the above suggestion as to the mode of glutamate oxidation, cell homogenates were allowed to respire added succinate or glutamate both in the presence and absence of the inhibitor. Respiration over a 2 hour period is shown in FIGURE 14. TABLE 8 gives the $Q_{O_2}(N)$ values calculated from the slopes of the O_2 uptake lines in the presence and absence of the inhibitor and the degree of inhibition caused by the presence of this compound.

TABLE 8

INHIBITION OF RESPIRATION BY MALONATE

Substrate	$Q_{O_2}(N)$		Percent inhibition *
	no malonate	malonate	
endogenous	100	82	18
succinate	191	118	38
glutamate	282	145	49

* as compared to the corresponding treatment in the absence of malonate.

The inhibition of endogenous respiration caused by malonate would indicate that at least a portion of the endogenous respiratory activity was passing through succinate. This level of malonate (200μM/flask) was evidently not sufficient to block succinate oxidation completely though the increase

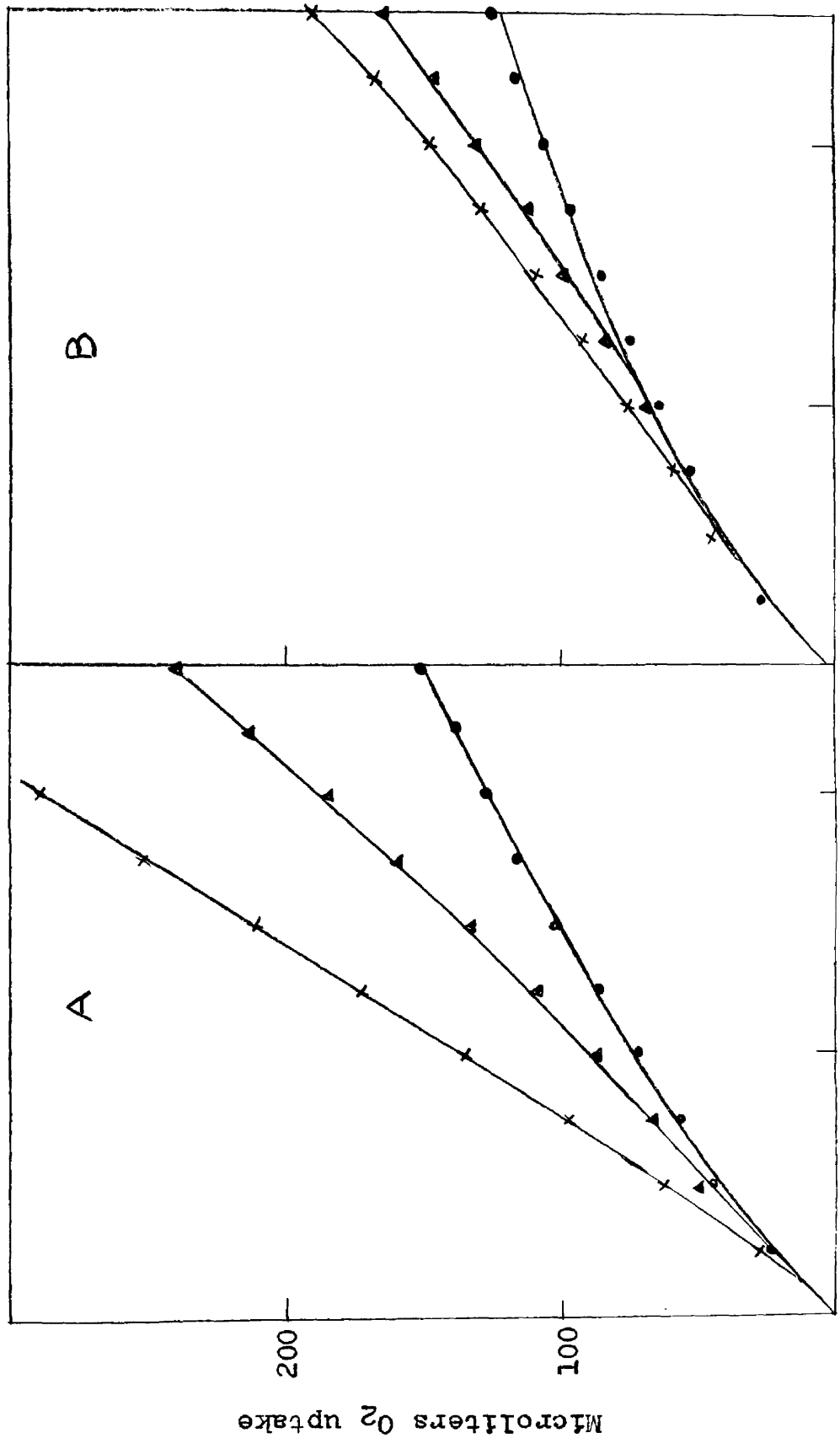

Fig. 14 Oxygen uptake by 15.5-hour homogenate (0.55 mgm. N/flask). A-no malonate, B-200 μM malonate/flask. Endogenous ●——●, succinate 5 μM ▲——▲, glutamate 5 μM ×——×.

in inhibition over that observed in the endogenous control tended to confirm the site of malonate action. More striking was the intense inhibition of glutamate oxidation in the presence of malonate. These data suggested that the oxidative steps in the pathway from glutamate to succinate were very rapid and depended upon the rapid removal of succinate by further oxidation. It is suggested that the failure to remove succinate in the presence of inhibitor slowed down the initial steps in the oxidative process thus giving the overall picture of an increased inhibition.

In the absence of malonate at the end of 3 hours the respiration rate with added succinate (5μM) decreased and was approximately equal to that in the control (endogenous) vessels. A similar decline in rate was noted with glutamate added at the 5μM level. The difference in oxygen consumed (O_2 uptake $_{\text{glutamate}}$ - O_2 uptake $_{\text{succinate}}$) was approximately 120μl. whereas 112μl. was the calculated difference for the conversion of 5μM glutamate to 5μM succinate. The oxidation of succinate was far from complete; only about 25 percent of the calculated oxygen had been consumed when the rate with added succinate declined to the level of that in the control vessels.

The data of Cochrane and Peck showed that variations in permeability occurred as the mycelium of S. coelicolor aged (28). Other work with whole cells indicated that 1 and 2-day cells were able to carry out transamination reactions in which α-ketoglutarate was one of the reactants, a fact which

strongly suggested that the compound was able to gain entrance into older cells. In the course of other work where older mycelium was used it became evident that α-ketoglutarate oxidation could, in fact, be demonstrated directly (see section on pigment production). TABLE 9 contains data derived from a number of experiments which show very clearly the effect of mycelial age on α-ketoglutarate oxidation.

TABLE 9

INFLUENCE OF THE AGE OF THE MYCELIAL HOMOGENATE ON THE DEMONSTRATION OF α-KETOGLUTARATE OXIDATION

Substrate	Respiration with homogenate of age *					
	15.5	17	18	21	22	22
endogenous	449	490	480	384	364	334
α-ketoglutarate	461	492	486	558	490	485

* µl. O_2 utpake over 120 minutes/mgm. N. Homogenate age in hours.

A suggested interpretation of the above data is as follows: the oxidation of α-ketoglutarate can be carried out by these organisms although, because of permeability difficulties, the direct demonstration of this reaction is not always successful. However, when the organisms are supplied with glutamate, a postulated precursor of α-ketoglutarate and a compound which readily penetrates into the cell, the organism oxidizes the glutamate and behaves towards

malonate inhibition as one would predict assuming that the oxidation occurred as follows:

glutamate → ∝-ketoglutarate → succinate → end products

It is interesting to note the fact that the glutamate oxidation rate (even beyond the point of complete glutamic dehydrogenase activity) is just as high as that for succinate. These data suggest that the potential for ∝-ketoglutarate oxidation must be of the same or a greater order of magnitude than that which exists for succinate.

Pigment Production and Tyrosine Oxidation

Since the original work of Taylor and Decker (92) a number of attempts have been made to correlate biochemical properties of the actinomycetes with pathogenicity. The work of Vaisey (102) indicated that the brown ring test (92) was probably only a manifestation of melanin production arising as the result of tyrosinase activity.

An attempt was made to measure the formation of melanin by the method of Hollis (56) which involves the gravimetric estimation of $FeCl_3$ precipitatable substances. Under the experimental conditions used, however, blank values were high and replicates showed poor agreement. Other workers have measured the tyrosinase reaction by colorimetric methods (57, 90). To investigate the possibility of this method dilutions of a PM culture filtrate which showed intense blackening were made (with uninoculated PM as the diluent), and the

optical densities of the resulting solutions determined at a series of wavelengths using the Coleman spectrophotometer. The results of these trials are given in FIGURE 15.

These data show that there was a satisfactory straight-line relationship between optical density and pigment concentration at a number of wave lengths. The results at 540 mμ showed good sensitivity and agreement with Beer's Law. This wave length was used to compare pigment formation by a number of cultures.

A series of pathogenic (prefix P) and non-pathogenic (prefix S) actinomycete cultures were grown for 6 days in PM medium, the growth removed, and the culture filtrate diluted with uninoculated PM medium, and the optical density at 540 mμ determined. The results of these determinations are shown in FIGURE 16, and suggest that a very real difference in physiology exists between these forms. Chromatographic analyses of culture filtrates however revealed that tyrosine disappeared from the medium in the absence of detectable pigment production.

Lerner has indicated that a number of pathways exist for tyrosine oxidation (65, 66). In animal tissues tyrosine may undergo transamination with α-ketoglutarate, and the keto acid produced may be further degraded to yield acetoacetic and fumaric acids (66). In the true tyrosinase reaction, tyrosine is oxidized through dihydroxyphenylalanine (dopa) to an indole-like intermediate and finally polymerized to melanin (66). This latter reaction has been studied in some

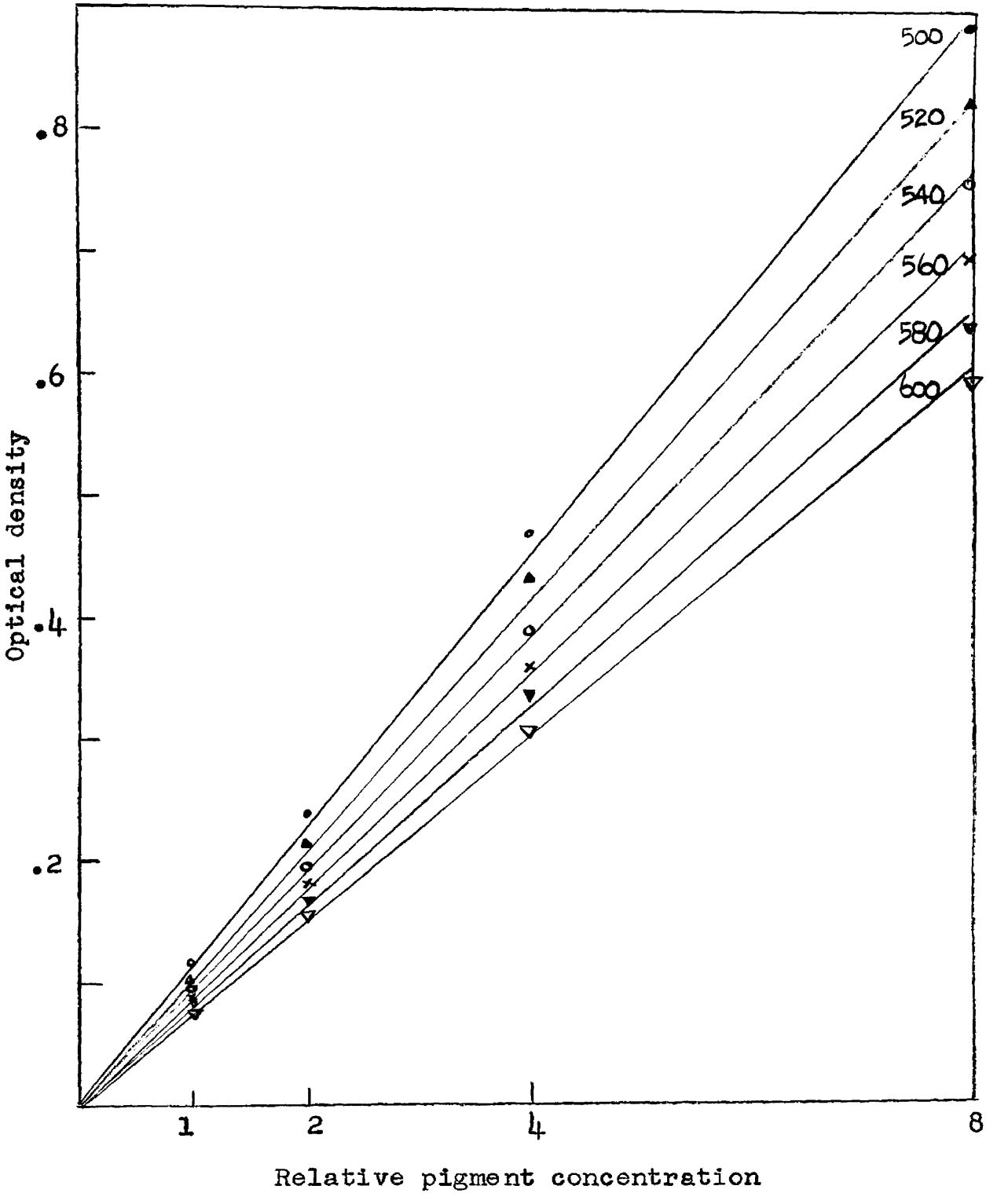

Fig. 15 Relationship between optical density and pigment concentration at a number of wavelengths.

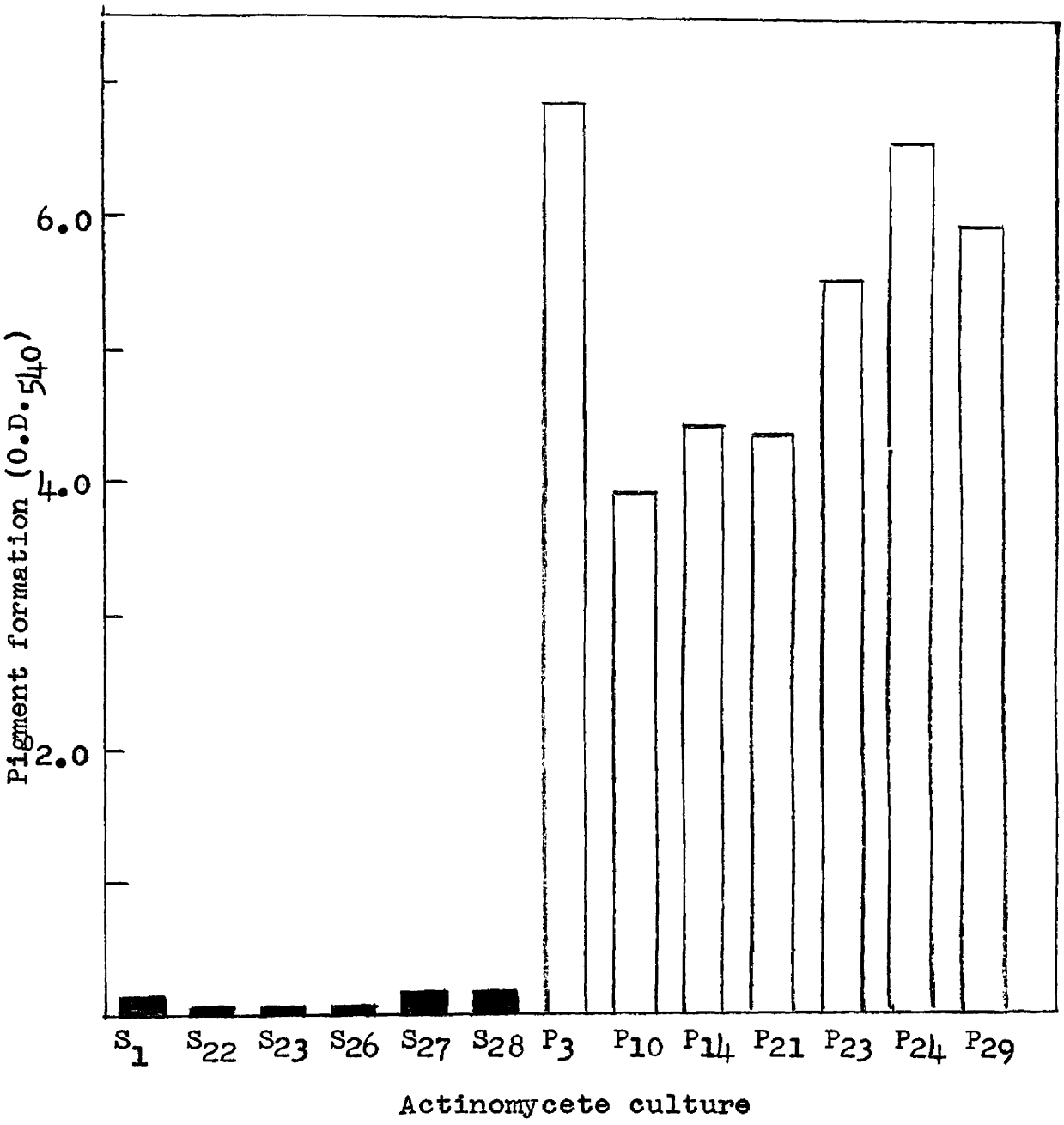

Fig. 16 Pigment formation by a group of non-pathogenic (S) and pathogenic (P) actinomycete cultures.

detail, and the data reveal that the initial step in the reaction (tyrosine ⟶ dopa) is slow and may be rate-limiting; however, once catalytic quantities of dopa have accumulated the oxidation proceeds at a much increased rate.

The oxidation of tyrosine, alone and with the addition of α-ketoglutarate or catalytic quantities (5μM/flask) of dopa, was followed using homogenates from a pigment producing strain P_{29}. Tyrosine was added as a suspension of crystals (0.25 ml./flask) and α-ketoglutarate was added at the rate of 50μM/flask. The results obtained are shown in FIGURES 17 and 18.

These data indicate that the oxidation of tyrosine and α-ketoglutarate apparently proceeded independently, since the uptake observed in the presence of both substrates very closely approximated the sum of the individually observed values. Contrary to expectations the addition of dopa did not stimulate tyrosine oxidation, but rather tended to prolong the induction period. A more significant point, however, is that the oxidation of tyrosine + dopa proceeded at a rate very similar to that of tyrosine alone - a condition to be expected if tyrosine oxidation occurred through dopa and the concentration of the former were not limiting. No explanation can be advanced for the prolonged induction period observed with dopa. The preparation used was a racemic (DL) mixture. Lerner (66) reported that this may not be as active as the L form. Chromatographic analyses of the contents of the Warburg vessels revealed negligible glutamate formation

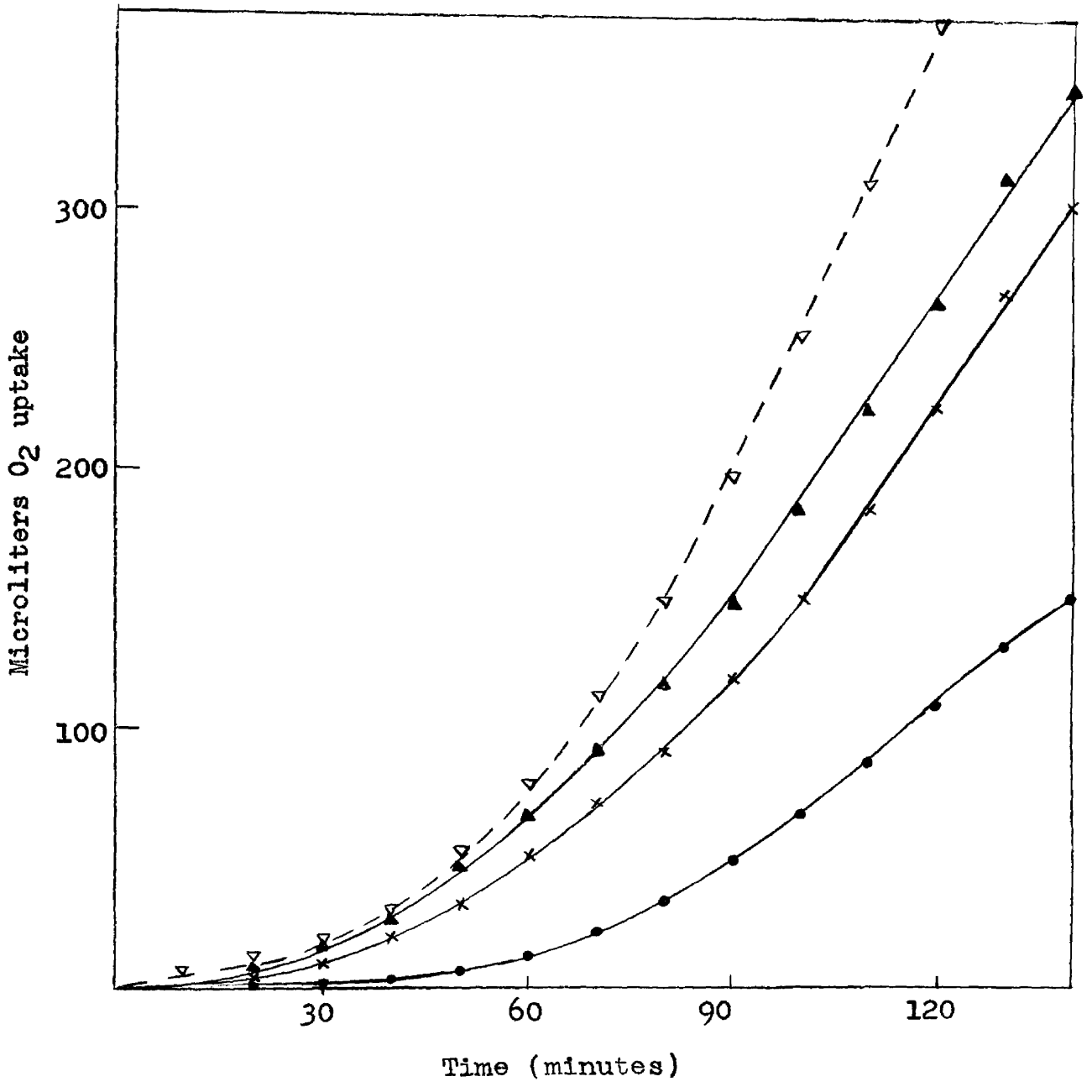

Fig. 17 Oxygen uptake by 22-hour homogenate (0.63 mgm. N/flask). Dopa ●——●, tyrosine ▲——▲, tyrosine plus dopa ×——×. Calculated value for the simultaneous oxidation of tyrosine plus dopa ▽---▽. Endogenous uptake (209 μl. at 120 min.) subtracted.

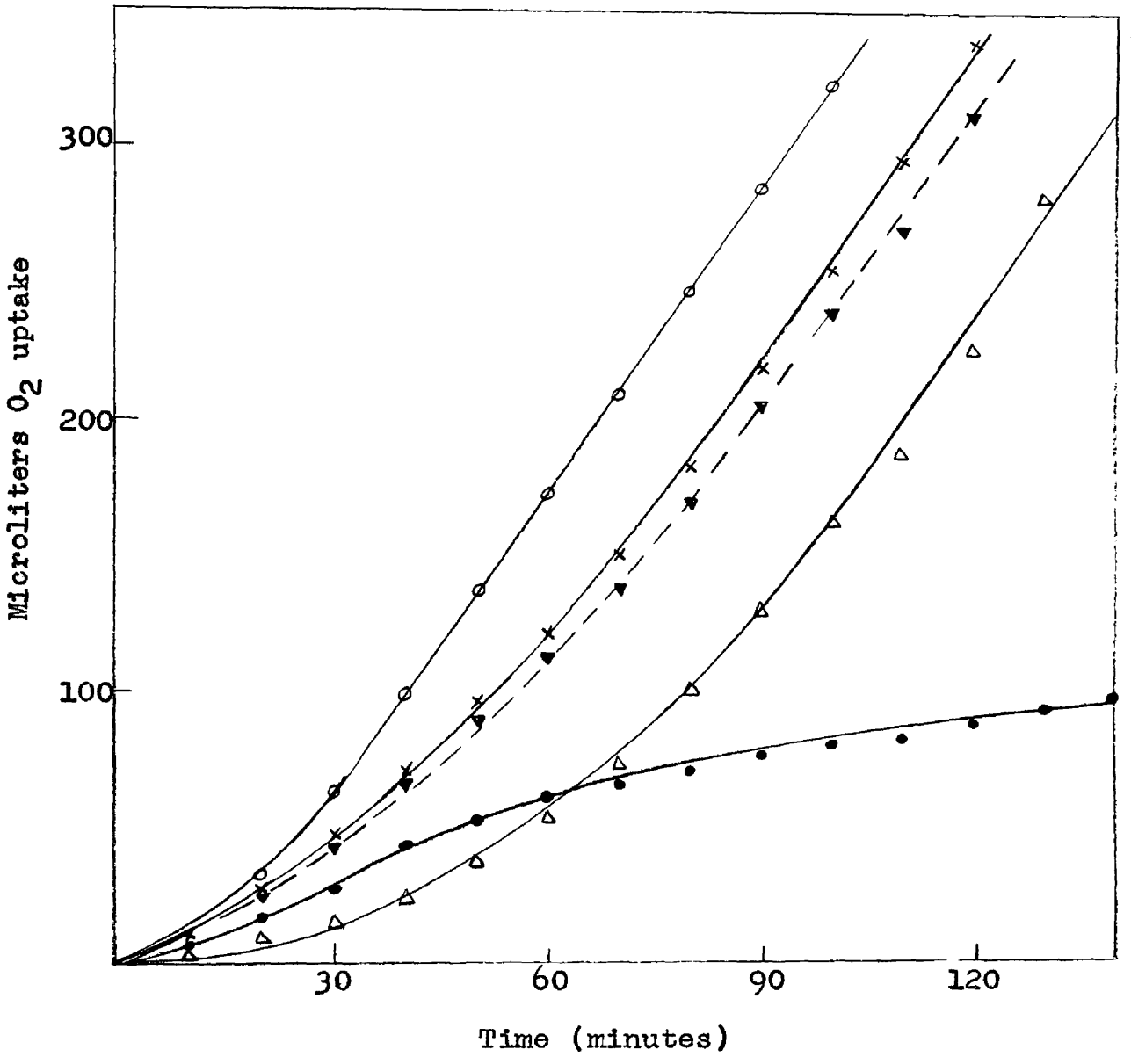

Fig. 18 Oxygen uptake by 22-hour homogenate (0.70 mgm. N/flask). ∝-ketoglutarate •——•, tyrosine △——△, ∝-ketoglutarate plus tyrosine ×——×, and glutamate o——o. Calculated value for the simultaneous oxidation of tyrosine plus ∝-ketoglutarate▼- - -▼. Endogenous uptake (255 μl. at 120 min.) subtracted.

in the α-ketoglutarate + tyrosine flasks. Similar negative data were obtained with cell extracts. Under similar conditions alanine and valine could be shown to contribute to glutamate formation. The data support the conclusion that the oxidation of tyrosine occurred chiefly by way of melanin formation. Considerable pigment formation occurred in these vessels containing tyrosine or dopa, but was most advanced in the flasks which also contained added α-ketoglutarate.

A similar experiment was carried out with a non-pigment producing strain S_{29}. This organism also oxidized tyrosine, but no oxidation of dopa could be shown over a 2 hour observation period. No melanin formation was observed in any of the Warburg vessels. Further work is planned to determine whether this failure to oxidize dopa is characteristic of all the non-pigment forming strains.

Discussion

Recent reports on manometric investigations using S. griseus (15, 46) and S. nitrificans (72) reveal that these organisms possess a pattern of respiratory activity similar to that shown in the present work with S. scabies. The present data are consonant with the operation of a Kreb's cycle in the terminal oxidative pathway although manometric data alone constitute insufficient evidence to advance as proof of this (28).

Endogenous respiratory activity was quite high, a finding in agreement with the results of others working with

actinomycetes (15, 28, 72), and was accompanied by an increase in NPN as determined by partition with TCA. This latter observation supported the contention drawn from R.Q. data that nitrogenous reserves constituted at least part of the endogenous substrate. The pattern of endogenous activity consistently observed (i.e. the rapid initial rate followed by a lower, relatively constant rate) suggests that the endogenous reserve material may be heterogenous in nature. Attempts to reveal a change in total carbohydrate after respiration in buffer, however, failed to show any consistent decrease.

The short starvation period employed in much of this work as an aid in reducing autorespiration has also been found valuable with S. nitrificans (72). This technique coupled with the use of large inocula and young mycelium resulted in the satisfactory demonstration of exogenous respiration with most substrates tested. Failure to observe the latter conditions, however, resulted in the growth of mycelium with which exogenous respiration was difficult or impossible to demonstrate.

In those experiments in which exogenous respiration terminated during the observation period it was evident that the oxidations were incomplete. Low NH_3 recoveries during amino acid oxidation observed by other workers (10, 11) were interpreted as indicating oxidative assimilation. Definitive evidence on this point was often not available because of the long lag periods encountered and the fact that the observation

period was necessarily restricted in "resting cell" investigations. The use of high substrate concentrations to obtain increased oxidation rates also precluded further observations on this point.

Cellular impermeability to added substrates is a source of difficulty in much respiration work (28, 45, 62, 82). Umbreit (100) indicated that citrate, isocitrate, oxalosuccinate, and α-ketoglutarate were apparently not metabolized by intact cells of E. coli whereas compounds of the Kreb's cycle below α-ketoglutarate did penetrate and could be shown to be oxidized by cell suspensions. Gilmour et al. (46) observed the failure of S. griseus to oxidize succinate, malate or fumarate unless the substrate concentration was very high or the mycelium was preincubated with the substrate. In the present work α-ketoglutarate oxidation has been difficult to demonstrate. Undoubtedly, cellular permeability is a function of both the organism and the cultural conditions employed (58, 99). Kann and Mills, for example, were able to show α-ketoglutarate oxidation, but were unable to demonstrate malonate inhibition with intact cells of Pasteurella tularensis (62).

The lag periods occurring before maximum oxidative activity is reached have been the subject of numerous investigations. Back and Mitchell (5) explained their observations on the basis of "temporary impermeability" while other workers indicated that the process involved was an energy-requiring "adaptation" (3). Gerhardt et al. (45) suggested

several experimental approaches to the problem (variation of pH, esterification of substrates, alteration of cellular permeability and the use of extracts). Data obtained using cell extracts will be reported in a later section although it is evident that such observations do little to confirm or deny the concept of "adaptive transport" proposed by Williams and Wilson (105, 106).

During the malonate inhibition experiments attempts were made to demonstrate succinate accumulation by paper chromatography (32) when cell homogenates were incubated with glutamate and malonate. These experiments were not successful. Similar observations were reported by Marr et al. (68) who concluded that the initial step in glutamate oxidation was very sensitive to the presence of inhibitors.

The oxidation of α-ketoglutarate obtained with older cell homogenates presents an apparent anomaly in that the respiration rate was much lower than that of either glutamate or succinate. Similar observations have been made with P. tularensis (62) although with this organism the rate of succinate oxidation was also low. It is probable that these observations are simply a function of permeability since Gilmour et al. (46) have shown that progressively increasing concentrations of α-ketoglutarate resulted in correspondingly increased rates of oxidation by S. griseus suspensions.

CELL EXTRACTS AND DYE REDUCTION

The typical lags in the oxidation of a number of substrates by cell homogenates and the inability to distinguish with certainty between inductive and permeability effects prompted the investigation of mycelial extracts. A number of techniques are available for the preparation of cell extracts (58) and of these grinding with an abrasive (sand, glass or alumina) appeared to be most promising. It was recognized at the outset that negative results with such preparations might be of limited value since maximum activity is generally obtained only after some reconstruction (58, 82, 97).

Experimental Methods and Results

Grinding in a mortar with sand and in a glass homogenizer with alumina was tried; the latter appeared to be the most reliable, and was used throughout this work. The mycelium was harvested and washed with saline or KCl solution (0.02 percent) in the centrifuge, then ground for 10 minutes with an equal volume of alumina in a glass homogenizer chilled in an ice bath. The disruptate was extracted with phosphate buffer (pH 7.0, 0.025 M) and centrifuged at approximately 1600 XG for 5 to 10 minutes. The extracts were maintained in an ice water bath until used.

A number of attempts were made to obtain cell extracts capable of bringing about oxygen uptake in Warburg vessels. Preparations obtained with glass powder or alumina do show

a small endogenous uptake but little or no stimulation was observed in the presence of added substrate. Additions of adenosine triphosphate and diphosphopyridine nucleotide had no stimulatory effect. Methylene blue was added in an effort to by-pass a possible limiting factor in the terminal oxidative system (71), but no significant stimulation was observed. The addition of Mg^{++} ions was also without effect. Activity was observed only with extracts from young (1, 2-day) cells, and usually did not persist beyond 30 minutes after the equilibration period.

The failure of cell extracts to use oxygen as an electron acceptor prompted the use of an artificial carrier (33, 71). Following the suggestion of the work of Nickerson and Mohan (75) and Casida and Knight (20) the dye 2, 6-dichlorophenol indophenol (phenol-indo-2:6 dichlorophenol, B.D.H.) was chosen. Auto-oxidation of this dye is negligible (53) so that the necessity of carrying out the reactions in evacuated tubes (Thunberg technique) is avoided. The relatively low ability of the extracts to use O_2 as a terminal acceptor made successful use of the dye more likely.

Reactions were carried out in 12x75 mm cuvettes with a Coleman junior spectrophotometer. Phosphate buffer (pH 7.0, 0.025 M), substrate and extract were added, mixed by inversion, and the optical density of the mixture set to zero. The dye was then added, mixed by inversion, and the decolorization observed by following the decrease in optical density at 600 mμ ($\Delta O.D._{600}$) over a period of time. The dye concentra-

tion initially used was 0.002 percent (final concentration). Some reactions were carried out with a final dye concentration of 0.013 percent so that a more favorable portion of the spectrophotometer scale was employed.

As in the manometric work it was apparent that young cell material would produce the most active extract. One or 2-day cells gave active preparations but no activity could be demonstrated with extracts from 4-day cells.

Typical dye reduction results are shown in FIGURE 19. The results of a number of trials indicated that succinate, fumarate, and citrate stimulated dye reduction over the endogenous rate. No increase in rate could be shown for acetate. Attempts to "spark" acetate oxidation by the use of C_4 acids (succinate, fumarate) were not successful.

The activity of extracts may be compared by measuring the change in optical density during the initial stages of the reaction. (This is essentially the method of Englesberg and Levy). TABLE 10 shows that an approximately straight-line relationship exists between this measurement and the amount of extract used in the reaction. A maximum is reached however, and increasing amounts of extract then no longer cause proportional increases in optical density change.

A number of attempts to show dehydrogenase activity with α-ketoglutarate were unsuccessful. The interpretation of other data (e.g. glutamate oxidation) hinged upon successful demonstration of activity towards this substrate so extracts were fortified with DPN in an effort to bring about

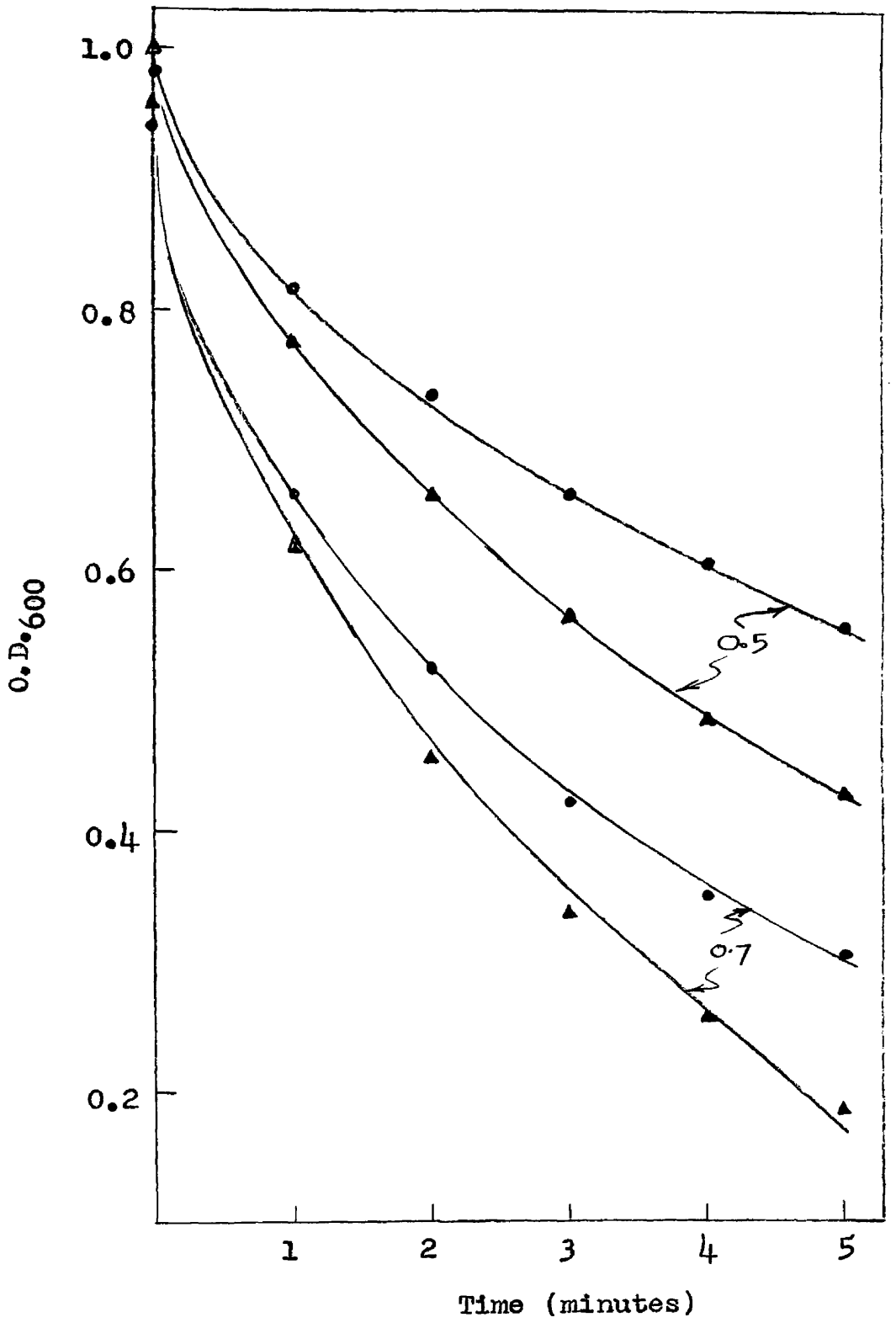

Fig. 19 Reduction of 2,6-dichlorophenol indophenol (0.02%) by 0.5 and 0.7 ml. of extract from 44-hour mycelium. Endogenous ●——●, succinate 10 μM ▲——▲.

TABLE 10

RELATIONSHIP BETWEEN THE AMOUNT OF EXTRACT AND THE RATE OF DYE REDUCTION

Expt.	Extract ml.	Δ O.D.$_{600}^{1-2}$ *	
		observed	calculated **
1	0.5	120	-
	0.6	136	144
	0.7	164	168
	0.8	170	192
2	0.3	139	-
	0.4	187	184
	0.5	208	230
3	0.3	108	-
	0.4	135	144
	0.5	185	180
4	0.5	39	-
	0.7	49	46
	0.9	56	59
	1.1	73	72

* change in optical density at 600 mμ between 1 and 2 minutes. ($x10^{-3}$)

** calculated from the observed value with the least amount of extract used.

activity against ∝-ketoglutarate. The results of such an experiment are shown in FIGURE 20, and indicate that the addition of DPN will allow for the demonstration of dehydrogenase activity with ∝-ketoglutarate. Addition of DPN also stimulates fumarate, malate, and glutamate dehydrogenation as shown in TABLE 11. With some extracts activity cannot be shown against these latter compounds without the addition of pyridine nucleotide.

Discussion

The failure to demonstrate appreciable O_2 uptake with cell extracts was not altogether unexpected; other investigators have reported similar difficulties (54, 72, 75). It is believed that further methods of reconstruction (58, 81,96) or the addition of protective protein (67, 81) may prove of value. Other experimental work has shown that phenazin derivatives may be more effective carriers than is methylene blue (28, 33).

Throughout the investigation it was apparent that the endogenous activity of extracts was very high. This was probably due in part to the significant quantities of amino acids in the extracts. Others have reduced such activity by dialysis (82) although this procedure generally adds to the reconstruction problems mentioned above(20, 29). Data obtained with cell homogenates showed that mycelium harvested before extensive reserve formation had occurred exhibited lower endogenous activity. The preparation of extracts from

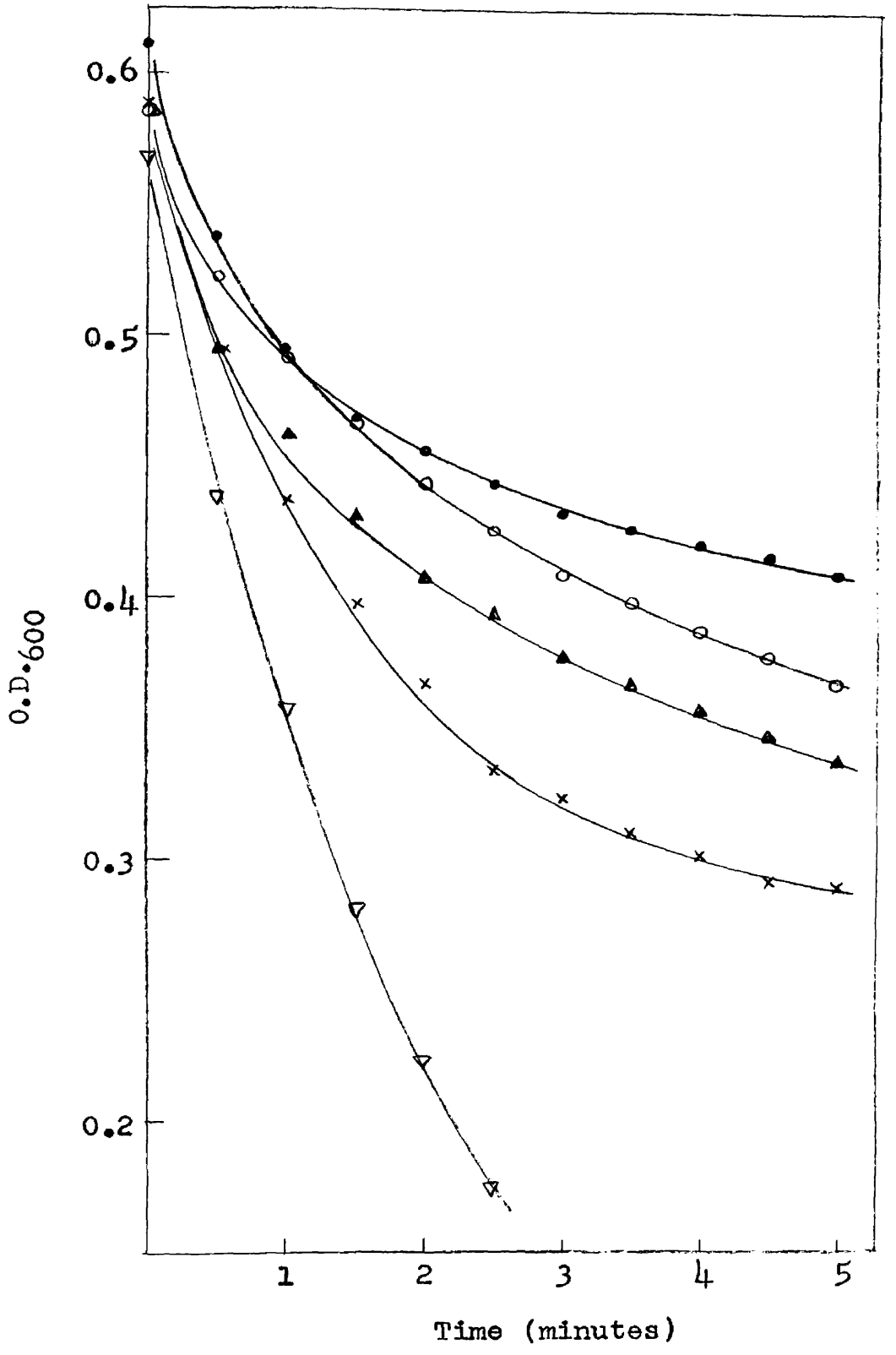

Fig. 20 Reduction of 2,6 dichlorophenol indophenol (0.013%) by 0.5 ml. of extract from 18-hour mycelium and the stimulation by DPN (500 μgm./3 ml. reaction mixture. Endogenous ●——●, endogenous+DPN ○ ○, glutamate ▲——▲, glutamate+DPN ▽——▽, α-keto+DPN ×——×.

TABLE 11

DPN STIMULATION OF DYE REDUCTION IN THE PRESENCE OF FUMARATE, GLUTAMATE, AND MALATE

Additions	$\Delta O.D._{600}^{0.5-1.5}$ when substrate was		
	fumarate	glutamate	malate
endogenous	.091	.083	.054
endogenous + DPN	.122	.113	.089
substrate	.088	.100	.051
substrate + DPN	.168	.155	.122

Reaction mixtures contained 0.2 ml. of 0.02% dye solution and 500 μgm. DPN where indicated. Amount of substrate: 15 μM of fumarate or malate, 60 μM of glutamate. Glutamate and malate reaction mixtures contained 0.5 ml. extract, fumarate reaction 0.3 ml. extract. Total volume made up to 3.0 ml. with phosphate buffer (0.025 M, pH 7.0).

very young mycelium represents another approach to the problem.

Cell extracts were able to bring about an immediate dye reduction in the presence of succinate; thus, the lag periods observed with homogenates cannot be interpreted as indicating adaptation, at least in the sense that succinic dehydrogenase is formed during the lag period. As discussed previously, however, these observations do not contradict an adaptive process concerned with the transport of substrate across the cell membrane.

The fact that dehydrogenase activity on glutamate was considerably greater than that observed with α-ketoglutarate suggested the possibility of demonstrating a net formation of the latter during glutamate dehydrogenation. To test this 2.0 ml. samples of cell extract were incubated in buffer alone and with added glutamic acid (100μM). To both reaction mixtures 2.5 mgm. of DPN were added. After 2 hours at room temperature the reaction was stopped and the mixtures deproteinized with TCA, and the supernates examined for keto acids by the method of Cavallini and Frontali (21). Chromatography of the phenylhydrazone derivatives in water saturated butanol indicated an increase in the α-ketoglutarate concentration in the mixture to which glutamate had been added. This experiment showed that the organisms were able to carry out the reaction glutamate $\longrightarrow$ α-ketoglutarate, and supports the oxidation scheme previously advanced.

The stimulation in the activity on fumarate caused by

the addition of DPN was probably a reflection of the cofactor requirement of the malic dehydrogenase enzyme in the extract. A similar DPN stimulation has been reported for extracts of a phycomycete (19); however, these extracts also showed malic dehydrogenase activity.

The disruption and extraction procedures used were admittedly inefficient. Similar alumina-grinding methods have been shown to result in recoveries of only 10 to 25 percent of the original N present in Azotobacter cells (52). These authors also observed that the method could not be standardized and that no definite fraction of the activity of the intact cell could be assurred in the extract. Lindstrom (67) noted that during alumina grinding much protein was lost by adsorption. Extracts prepared by the more efficient sonic vibration method contained a larger, reproducible amount of the original cell nitrogen and were shown to require less reconstitution for maximum activity (52).

TRANSAMINATION

Introduction

The actinomycete strains used in this work grew rapidly on a glucose-asparagine-salts medium. This observation suggested that the organisms were able to bring about considerable amino acid synthesis, an activity in which transamination is believed to play an important role (69, 70). A number of experiments were carried out to demonstrate the ability of these cultures to cause transamination.

Methods

In most of the work to be reported cell extracts were used. These were obtained by grinding the mycelium with alumina in a glass homogenizer as described in the section on dye reduction. Initially the reactions were carried out in evacuated Thunberg tubes, but essentially the same results were obtained by using small stoppered test tubes. Because of the ease of handling the latter they were used in most of the work. A complication in the use of Thunberg tubes is that during incubation considerable distillation occurs into the cap . With the small volumes employed considerable care must be taken to ensure this condensate is washed down and mixed with the rest of the tube contents in order to avoid possible distortion in the concentration relations when analyses are carried out.

Extracts were added to tubes containing NH_2-donor, keto acid and phosphate buffer (0.025 M, pH 7.0). Neutralized

solutions of amino and keto acids were added to establish final concentrations of 50 μM/ml. Extract was added at rate of 0.25 ml./ml. of reaction mixture unless otherwise noted. Tubes were incubated in a water bath at 37 C. Suitable controls were included as shown in the following chart:

TUBE	NH_2-DONOR	KETO ACID	BUFFER	EXTRACT
1	+	+	+	+
2	—	+	+	+
3	+	—	+	+
4	—	—	+	+
5	+	+	+	boiled

Tube 1 represents the complete reaction mixture; a sample was removed from this mixture at zero time and is designated as tube 1^{o} in subsequent analyses. Tube 5 contained boiled extract (5 minutes) and was included to ensure that the reaction was enzymatic in nature. Tubes 2, 3, and 4, in which the reactants were singly and simultaneously omitted, were included to establish with certainty the source of the product formed (see later discussion). At the end of the incubation period the reaction was stopped either by placing the tubes in a boiling water bath for 5 minutes or steaming the tubes for 10 minutes after which the contents were cooled and set aside for examination.

Reaction mixtures were analysed by paper chromatography with Whatman No. 4 paper and water-saturated phenol as the solvent (12). Development was carried out by the descending

method; the cabinet was held in an incubator at ≈ 26 C. Following chromatographic development the papers were suspended in front of a fan and allowed to dry at room temperature (42). Papers were sprayed with 0.1 percent ethanolic ninhydrin solution and the color was allowed to develop for 24 hours. When rapid results were required the papers were held in flowing steam for about 1 minute to allow for more rapid color development.

Where semi-quantitative results were desired, equal size spots (containing the amino acid) were cut out of the paper and the color extracted by shaking with 50 percent (v/v) aqueous ethanol. The optical density of the resulting solution was determined with a Coleman spectrophotometer at a wave length of 570 mμ. Blank values were obtained with extracts from a paper spot of equal area cut from the same sheet. Such a method has been shown to have an accuracy of ± 7 percent (60).

In this work it was found that within a chromatographic analysis excellent agreement was obtained between the optical density values of replicated spots. It was also found that the optical density was directly related to concentration for amounts of glutamic acid up to 13 μM/ml. The values, however, cannot be compared from day to day because of variation in the depth of color formed.

Results and Discussion

The ability of extracts from 1 and 2-day cells grown in PMG medium to transfer the NH_2-group to α-ketoglutarate from

alanine, glycine, and aspartic acid is shown in FIGURES 21 and 22. These extracts were of approximately the same activity; the slight differences with alanine and glycine were not believed to be significant. Some quantitative variation was usually noted even when similarly prepared extracts were used.

In most experiments it was found that in the control tubes substantial amounts of amino acids, particularly glutamic acid were produced. This made the evaluation of marginal activity difficult and established an absolute requirement for all the controls previously described.

In the control tubes it was noted that in the presence of α-ketoglutarate alone there was generally a greater final glutamate concentration than in the extract plus buffer control. This suggested that some material in the extract was supplying NH_2-groups for glutamate synthesis. In an effort to reduce this activity, 3.5 ml. of extract were dialyzed against 50 ml. of phosphate buffer (0.025 M, pH 7.0) in the cold for 2 hours with constant stirring. This dialyzed preparation and an undialyzed portion of the same extract were tested for glutamate formation with aspartate and alanine as NH_2-donors. Results of this experiment are shown in FIGURE 23.

These data show that the glutamate levels in the controls were lowered considerably by the dialysis treatment but that an apparent loss in activity also occurred. The results with the aspartate-glutamate system were rendered more decisive. Results with the alanine-α-ketoglutarate system were not

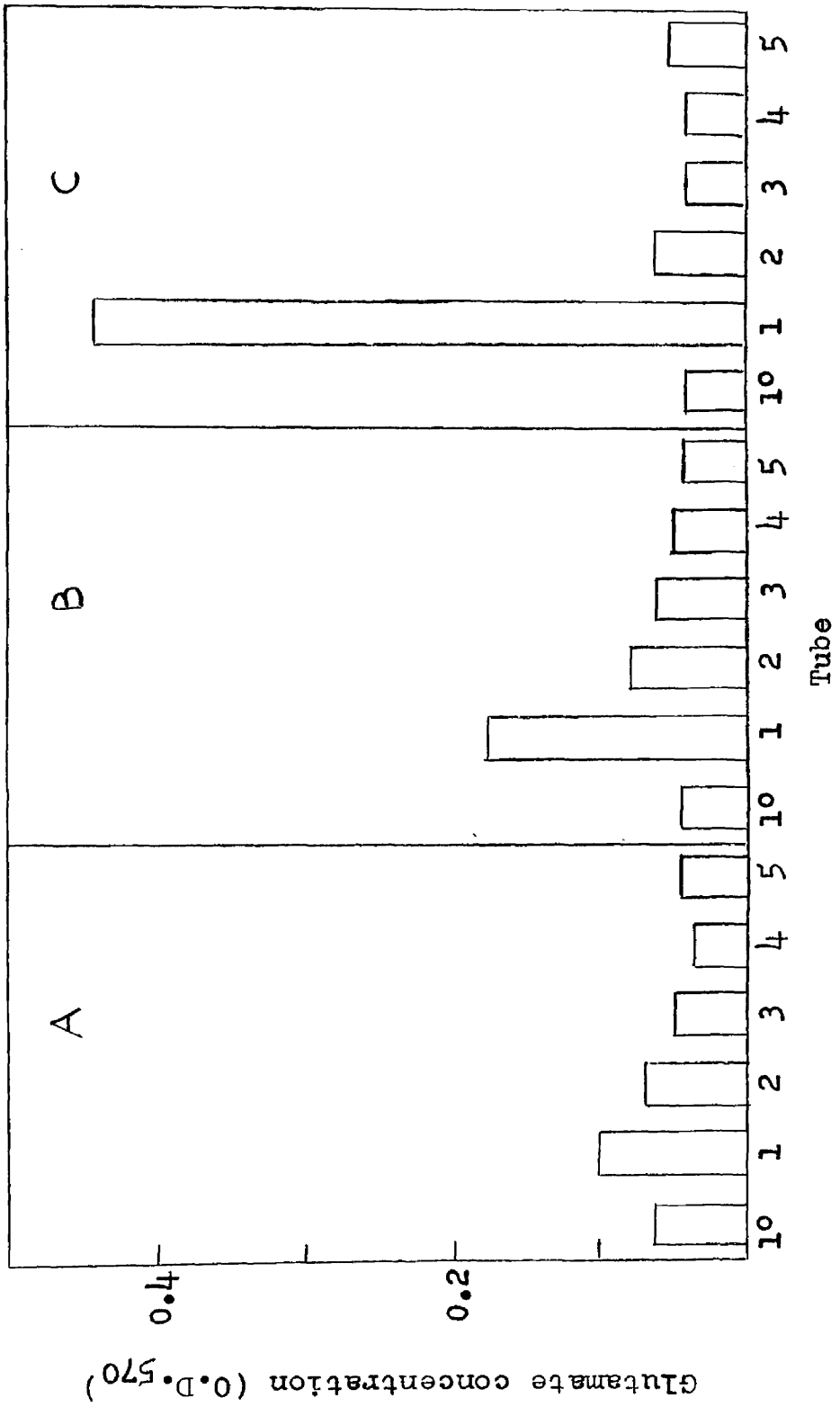

Fig. 21 Formation of glutamate by extract from 1-day mycelium (1.10 mgm. N/ml.) Reaction mixtures contained α-ketoglutarate and A-glycine, B-alanine, or C-aspartate. For tube contents see "Methods".

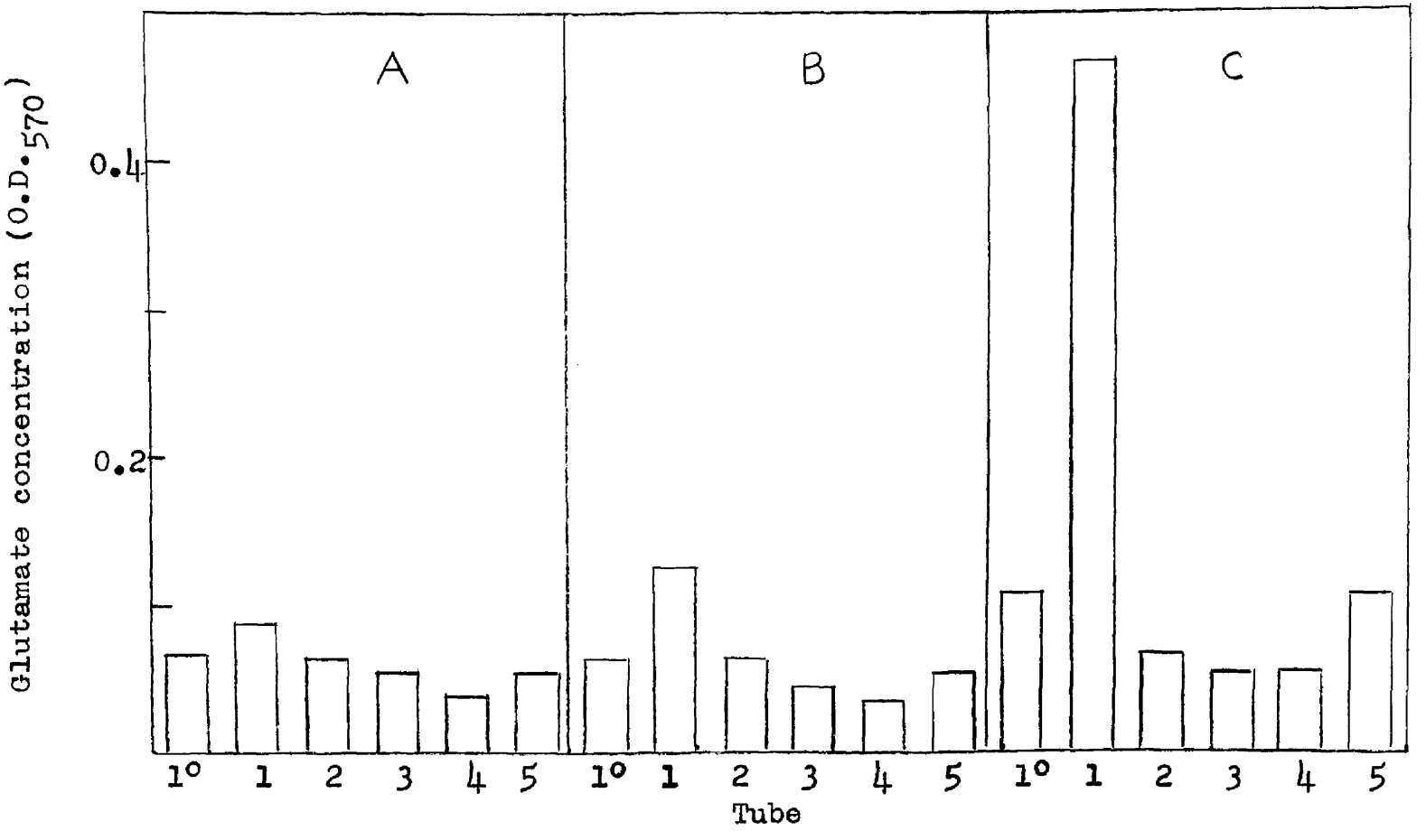

Fig. 22 Formation of glutamate by extract from 2-day mycelium (0.99 mgm. N/ml.). Reaction mixtures contained α-ketoglutarate and A-glycine, B-alanine or C-aspartate. For tube contents see "Methods".

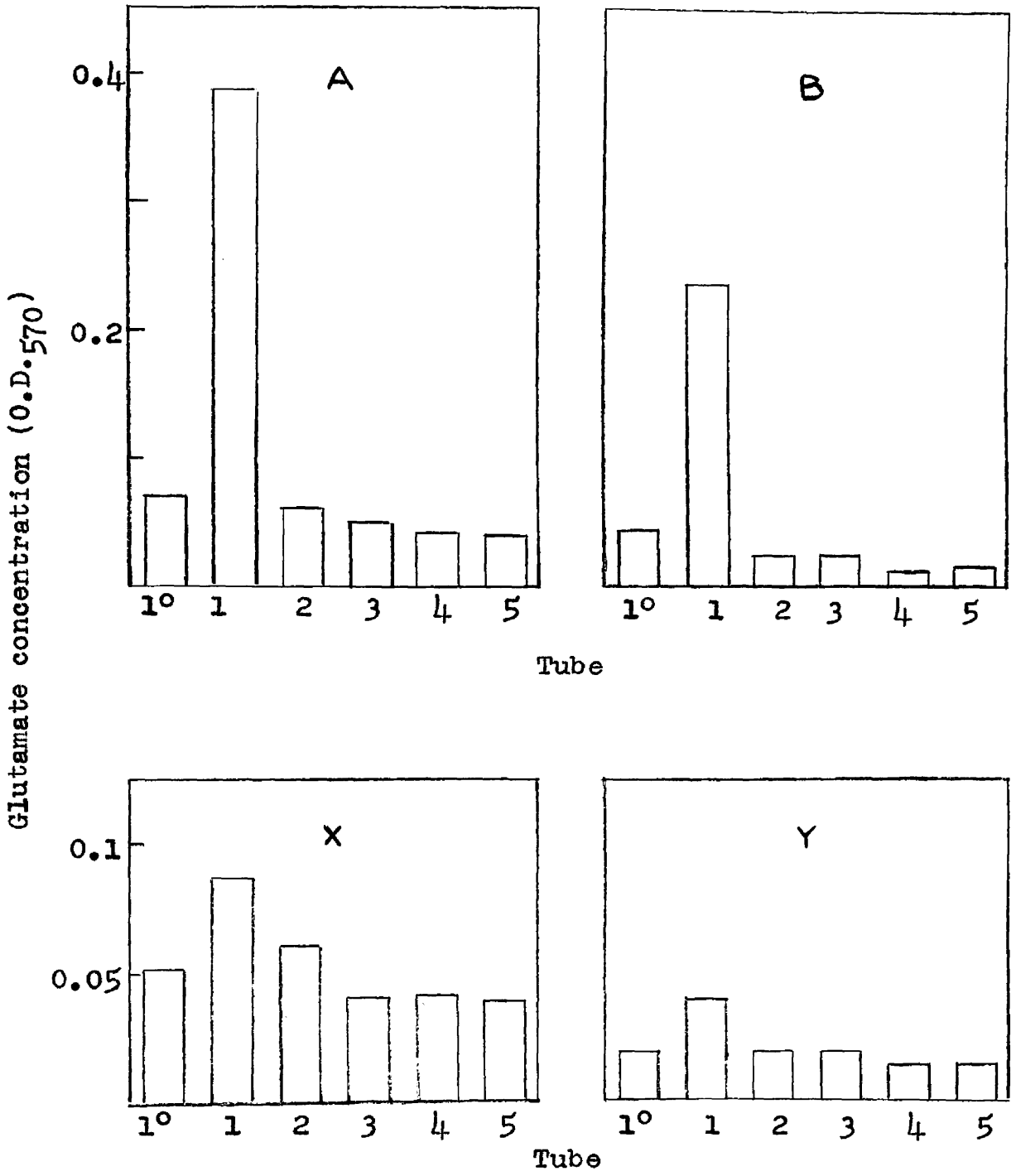

Fig. 23 Formation of glutamate from -ketoglutarate plus aspartate (A, B) or alanine (X, Y). Charts A, X show reaction with original extract (1.15 mgm. N/ml.) B, Y reaction with dialyzed extract (0.70 mgm. N/ml.). For tube contents see "Methods".

significantly changed. The latter system, however, was of considerably lower activity even before dialysis.

This dialysis treatment reduced the nitrogen content of the extract from 1.15 mgm./ml. to 0.70 mgm./ml., a reduction of approximately 40 percent. In several other experiments the protein (precipitatable) and non-protein (soluble) nitrogen were determined by fractionation with an equal volume of 20 percent TCA and subsequent nitrogen determinations by the Kjeldahl method. The NPN of several extracts was found to vary between 35 and 60 percent of the total nitrogen. To determine the nature of the components of the NPN fraction, samples were chromatographed in water saturated phenol, alone and with additions of known amino acid solutions. Results of these experiments showed that the extract contained significant quantities of ninhydrin-positive materials whose R_F values were comparable to those of glutamate, glycine, alanine, and leucine. Other ninhydrin-positive materials giving less well defined spots were also present. Evidently then, the extracts contained substances which, in the presence of α-ketoglutarate alone, could give rise to glutamic acid in the absence of an exogenous NH_2-donor.

Another fact which became evident from the chromatographic analyses was that non specific production of ninhydrin-positive materials occurred during the incubation period. In particular it was noted that spots corresponding to the positions of alanine and leucine were formed. (This observation is based on comparisons with the analyses of

boiled control and zero-time control tubes). An attempt was made to demonstrate the reaction:

glutamate + pyruvate $\longrightarrow$ alanine + α-ketoglutarate

but no significant alanine synthesis could be shown. It is suggested that this non-specific appearance of "amino acids" during incubation arose in part by the simultaneous proteinase activity of the extract during the incubation. Formol titrations carried out during the incubation of extract in buffer showed an increase in NH_2 groups occurred, an observation which supported the concept of proteolytic activity.

In dye reduction experiments it was found that in some cases the ability of the extract to reduce the dye was lost quickly when the extract was not kept in an ice water bath until used. Previous data showed that in some experiments on transamination the zero time control indicated glutamate synthesis (see FIGURE 23), and suggested that transaminase activity might be very rapid. An experiment was carried out to investigate the time course of the reaction between aspartate and α-ketoglutarate. The amounts of the additions to tube 1 were increased, and at intervals samples were removed, heated to stop the reaction, and analysed as previously described. Results of such an experiment are shown in FIGURE 24. With this particular extract the activity was essentially constant over the 2-hour incubation period. Other experiments showed that the activity may diminish after 1 hour, and emphasize the fact that considerable quantitative variation

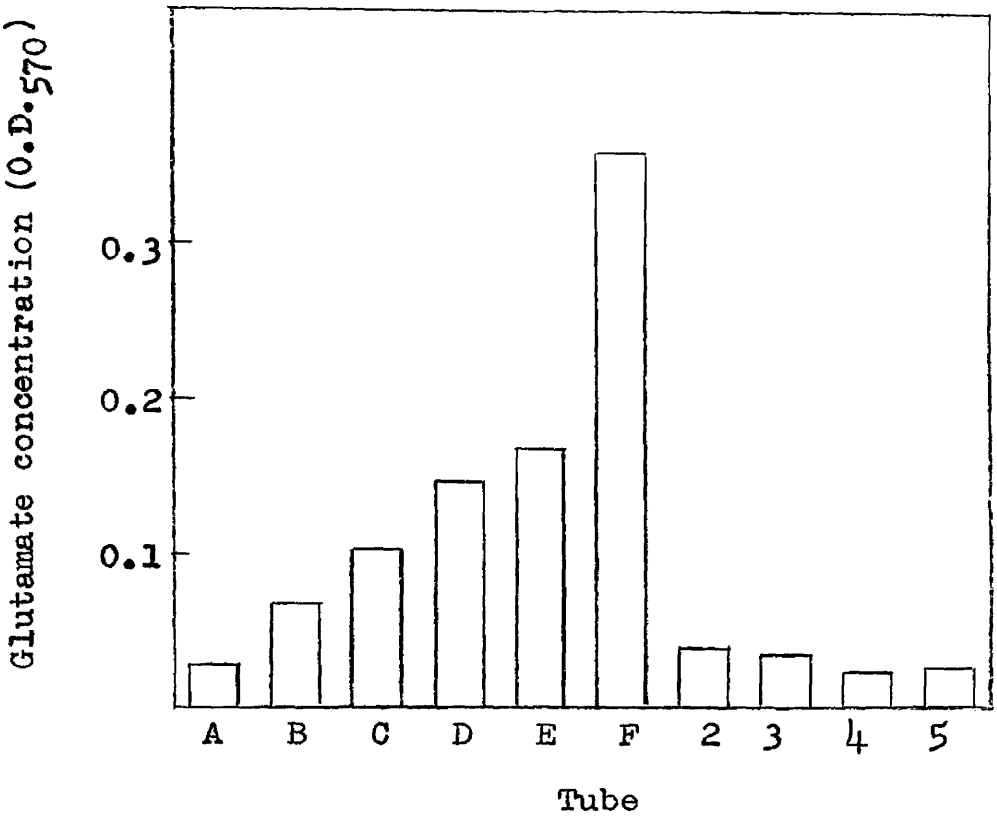

Fig. 24 Time course of the glutamic-aspartic transaminase reaction. Tubes A to F represent glutamate concentrations in the complete reaction mixture sampled at 0, 15, 30, 45, 60, and 120 minutes respectively. Bars 2, 3, 4, and 5 represent glutamate in the control tubes (see "Methods") sampled at 120 minutes. Extract from 1-day mycelium (1.19 mgm. N/ml.).

occurs between extracts. PLATE 1 shows a chromatogram of a similar experiment in which glutamate formation was determined at intervals up to 90 minutes.

All experiments discussed to this point were carried out with 0.025 M buffer, pH 7.0. To determine the effect of pH on enzyme activity, ground mycelium was extracted with saline, and the buffer solution in the reaction mixtures varied to obtain final pH values of 7.0, 7.5, and 8.0. Glutamate formation in the aspartate-glutamate system was observed, but no differences in activity were noted. It is suggested that a rather broad pH range of 7.0 - 8.0 exists for this system.

A number of neutralized amino acid solutions were prepared and tested for activity as NH_2 donors with α-ketoglutarate. The glutamate formation with these amino acids is shown in FIGURE 25. Transamination is indicated where the net glutamate formation in the complete reaction mixture (tube 1 − tube 4) is greater than the sum of the amounts formed when the reactants are added singly $\left[(\text{tube } 2 - \text{tube } 4) + (\text{tube } 3 - \text{tube } 4)\right]$. From these data it is apparent that aspartate, alanine, leucine, and valine can be used as amino donors. Threonine and lysine have negligible activity.

Transaminating activity was readily demonstrable with mycelial homogenates in lieu of cell extracts. Chromatographic analyses of the reaction mixtures in these systems were not as a rule as confused by non-specific "amino acid" formation although glutamate consistently appeared in the control tubes. Extensive work was not done with whole cells

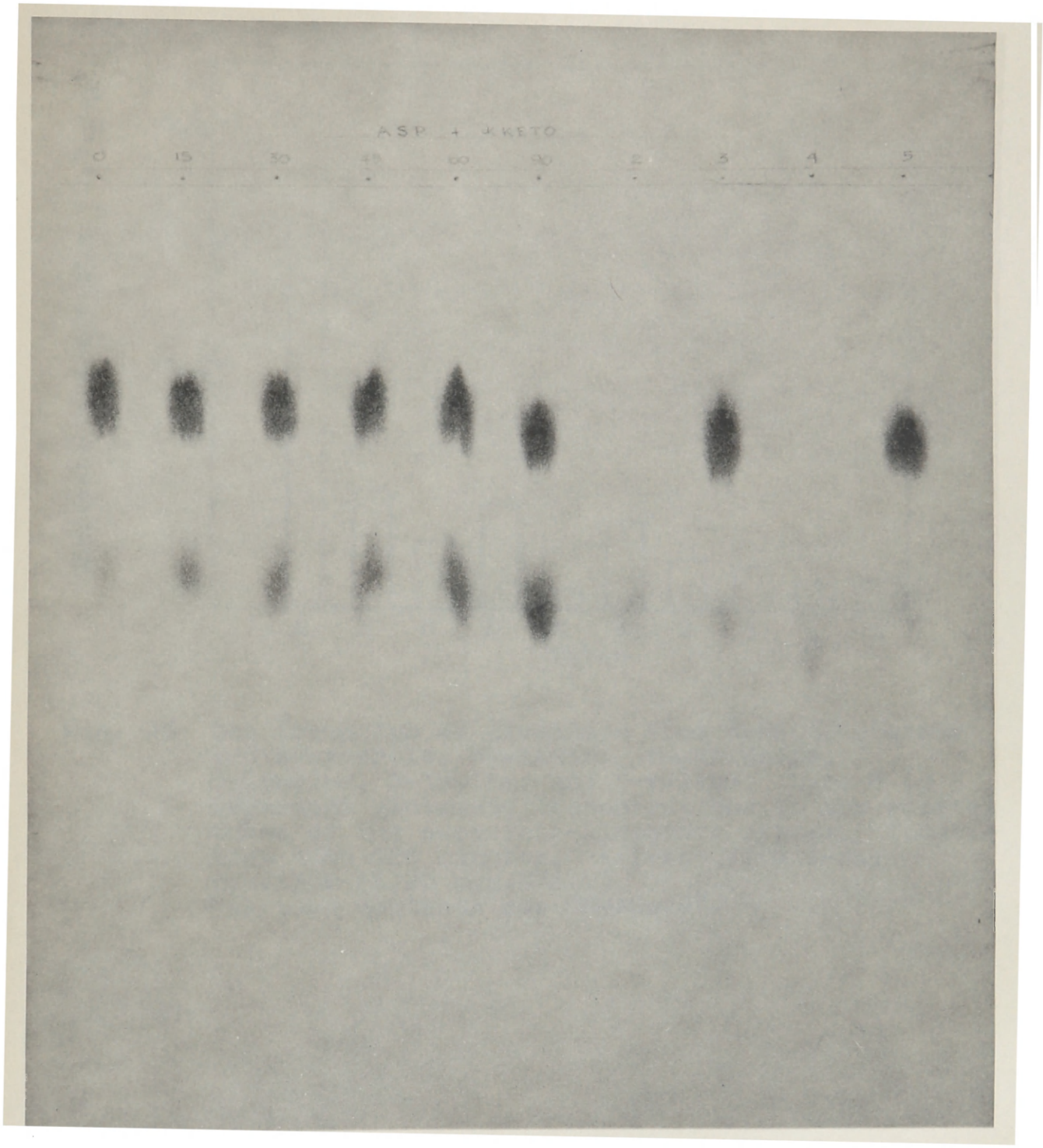

PLATE I. Chromatogram showing the time course of aspartic-glutamic transaminase activity by a mycelial homogenate. The first 6 spots represent analyses of the complete reaction mixture sampled at 0,15,30,45,60, and 90 minutes respectively. 2,3,4 and 5 represent the control tubes (see "Methods") sampled at 90 minutes. Upper spots are aspartic acid, lower spots glutamic acid.

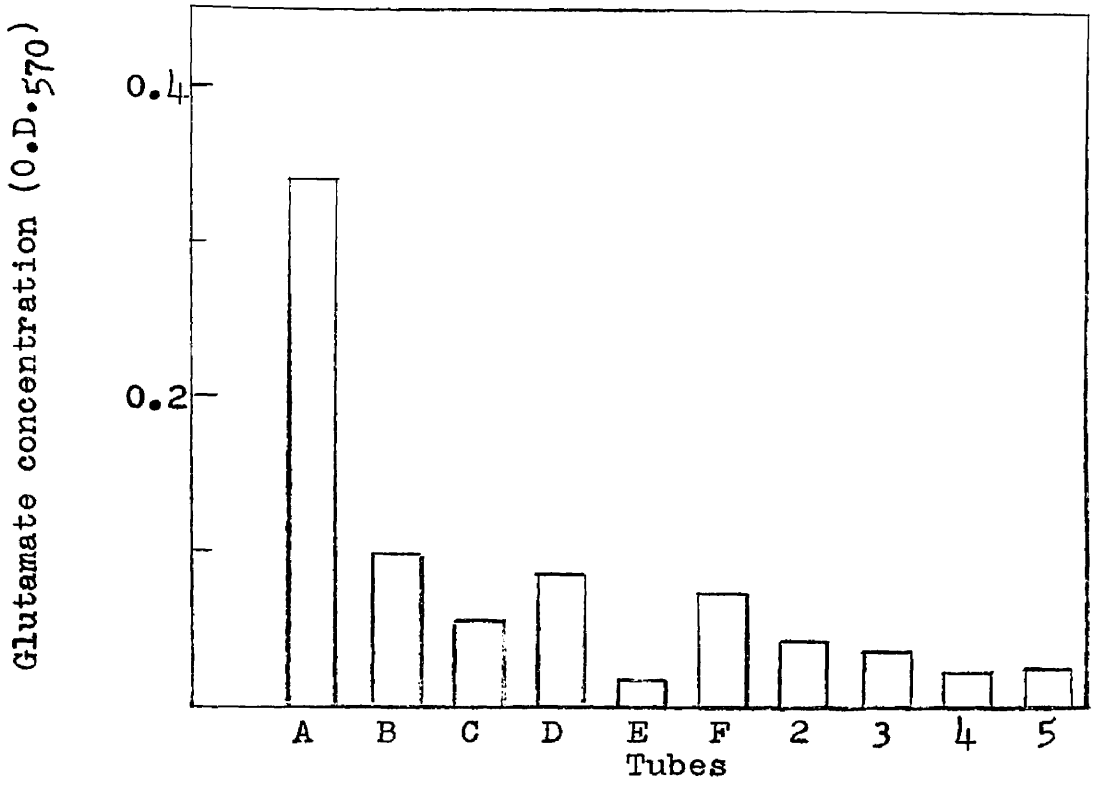

Fig. 25 The formation of glutamate from ∝-ketoglutarate and A-aspartic, B-alanine, C-threonine, D-leucine, E-lysine, and F-valine. Bars 2 to 5 represent glutamate concentrations in the control tubes of the aspartate-glutamate system. Incubation time 120 minutes. Extract from 1-day mycelium (1.20 mgm.N/ml.).
For tube contents see "Methods".

since it was believed that negative results would be subject to criticism in that they could arise because of cellular impermeability to one of the reactants.

The ability of the amides, asparagine, and glutamine, to act as NH_2-donors to ∝-ketoglutarate was investigated. Results of colorimetric analyses of glutamate concentration indicated that the combined presence of glutamine and ∝-ketoglutarate did not yield more glutamate than the sum of that formed in the control tubes in which these components were separately omitted. With asparagine the control tube in the absence of keto acid showed the formation of aspartate, but the latter was reduced in amount when ∝-ketoglutarate was added. These results suggest that asparagine is probably deaminated to aspartic acid which in turn transaminates with ∝-ketoglutarate to form glutamate in the complete reaction mixture. There is no good evidence for a direct transamination reaction with either glutamine or asparagine and ∝-ketoglutarate. PLATE 2 shows a chromatogram from a similar experiment carried out with a mycelial homogenate instead of cell extract. The reaction in which aspartate supplies the NH_2-group has been included as a control on the activity of the homogenate.

It was previously stated that alanine synthesis could not be demonstrated in reaction mixtures in which cell extracts were incubated with glutamate and pyruvate, although the opposite reaction (glutamate formation from alanine and ∝-ketoglutarate) could be demonstrated. With the more active

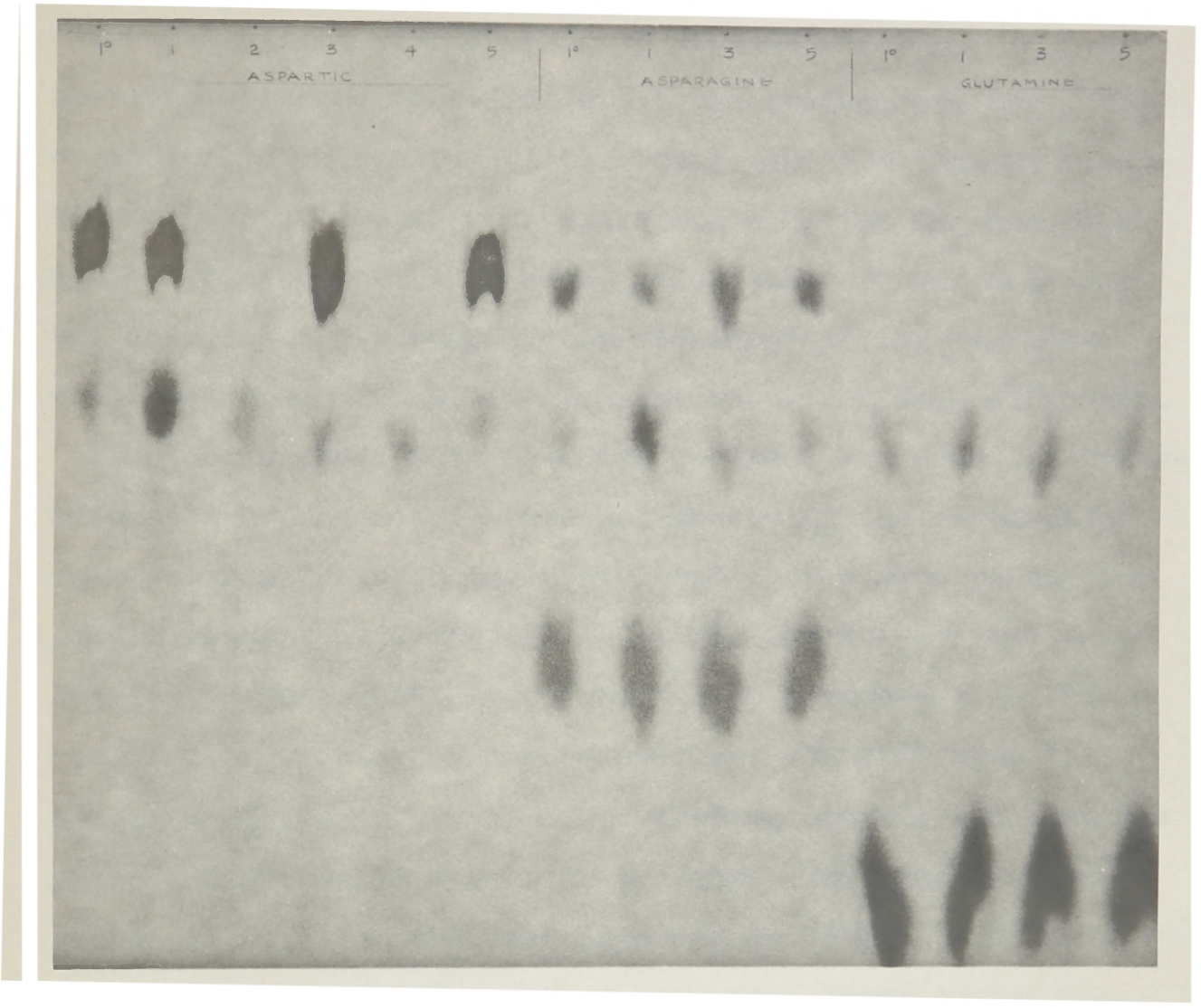

PLATE 2. Formation of glutamate from α-ketoglutarate and aspartate, asparagine, or glutamine by a mycelial homogenate. Tube contents are described in "Methods". Incubation time 90 minutes.

glutamate-aspartate system, however, it was possible to demonstrate the reaction in both directions. PLATE 3 shows these activities with an extract from strain P_{24}. In some previous experiments sodium ethyl oxalacetate was used instead of oxalacetate itself, but the ester gave conflicting results. No work was attempted with oxalacetate as the NH_2-acceptor in the presence of other amino acids since it was felt that the consistent glutamate "contamination" of the extracts would make unequivocal interpretation of the data difficult.

Other workers have obtained indirect evidence for transamination by manometric methods, observing the respiration when the reactants in the postulated system were singly and simultaneously oxidized (80, 87). To investigate the feasibility of this method of transaminase demonstration the respiration of starved homogenates was observed upon the addition of alanine, valine, or α-ketoglutarate and when the amino and keto acid were both present. The oxidation of glutamate was also observed in order to establish an activity level on the proposed reaction product. These particular amino acids were chosen because the one (valine) was only slowly oxidized, and the other (alanine) was oxidized only after an appreciable lag period. Aspartate was not investigated because the oxidation of this amino acid is almost as rapid as that of glutamate itself. The results of these experiments are shown in FIGURE 26.

In the valine-glutamate system toward the end of the observation period there was some indication of transaminase

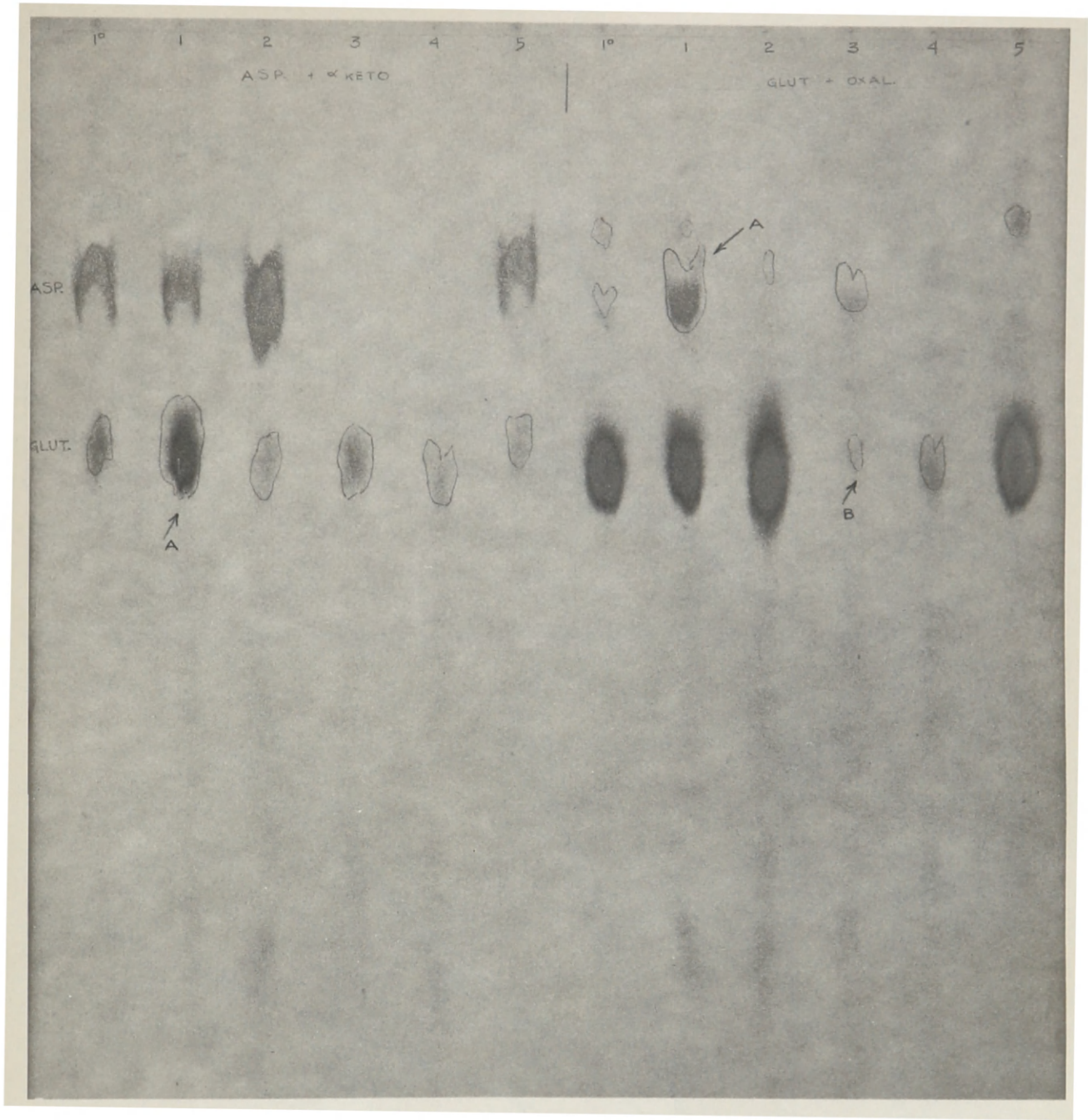

PLATE 3. Chromatogram showing glutamic-aspartic transaminase activity by a mycelial extract. The reaction between aspartate and ∝-ketoglutarate is shown on the left; the reverse reaction between glutamate and oxalacetate is shown on the right. Time 120 minutes. Arrows A indicate amino acids produced in the complete reaction mixture; B indicates the disappearance of endogenous glutamate in the presence of oxalacetate.

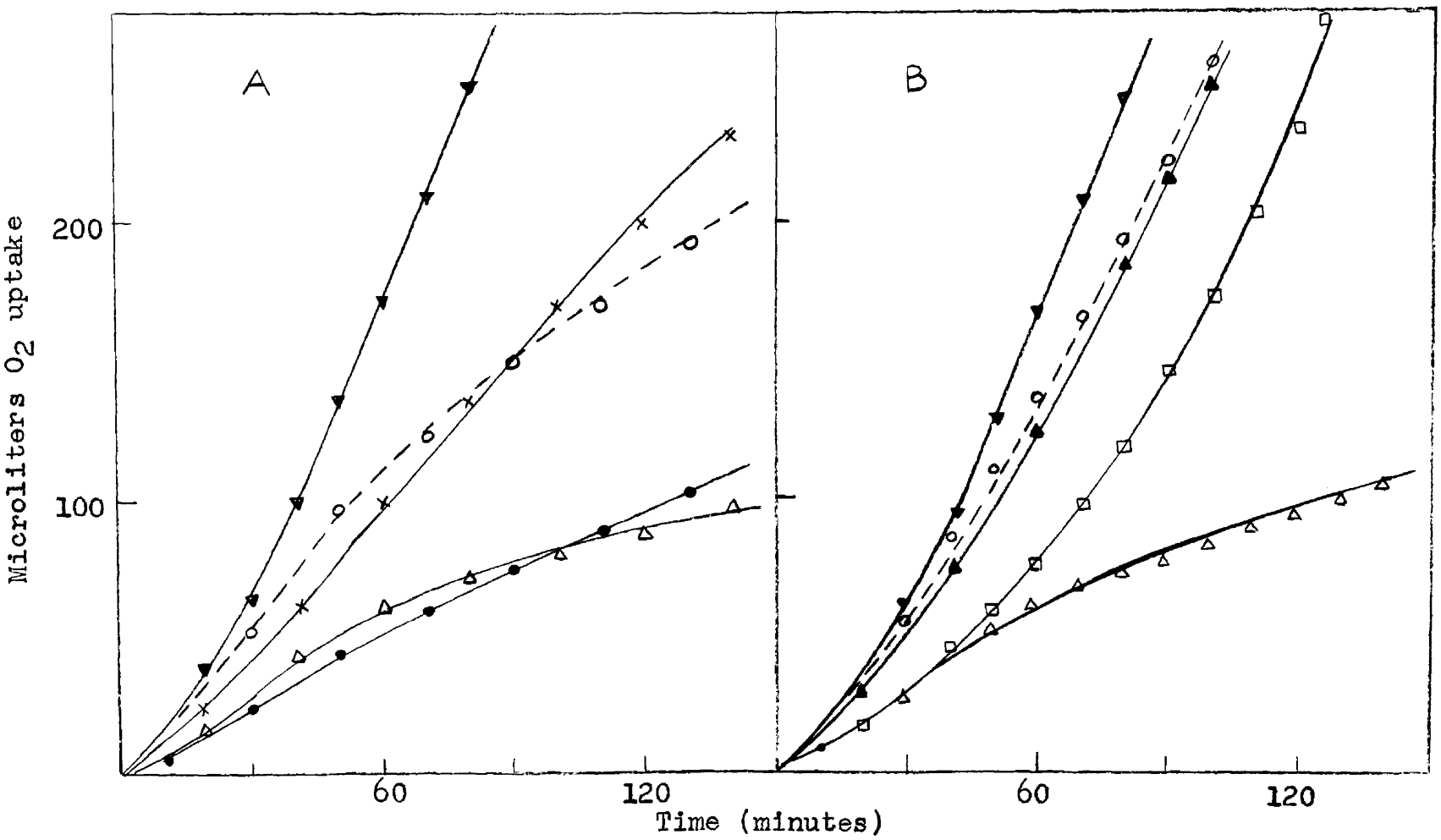

Fig. 26 Oxygen uptake by 22-hour mycelial homogenates. Glutamate ▼—▼, valine ●—●, α-ketoglutarate △—△, alanine □—□, valine+α-ketoglutarate: observed ×—×, calculated o---o; alanine+ α-ketoglutarate: observed ▲—▲, calculated o--o .
A-0.70 mgm. N/flask, endogenous 255 μl. at 120 minutes subtracted.
B-0.63 mgm. N/flask, endogenous 209 μl. at 120 minutes subtracted.

activity although the results were not conclusive. With the alanine-glutamate system, however, this method failed to reveal any transaminase activity. When the contents of replicate Warburg vessels were pooled and the supernatants analysed by paper chromatography glutamate formation was evident in both systems. It is apparent, at least for the reactions above, that the manometric method is a less sensitive device for following transaminase activity than is the method of determining end-product formation.

It was observed that some extracts were able to carry out transamination reactions, but possessed no dehydrogenase activity as judged by dye reduction. This was probably a reflection of the avidity with which cofactors were held by different enzyme systems. Other workers have shown that pyridoxal phosphate, the cofactor in transamination, may not be resolved from the apoenzyme even by extensive dialysis (41, 70, 85). Additions of pyridoxal hydrochloride (3μgm.) and ATP (5 mgm.) to the aspartate-glutamate system caused no stimulation, an observation which suggested that the cofactor level was not limiting. On the other hand, the addition of pyridine nucleotide in dehydrogenase experiments showed that in these extracts a cofactor for respiratory enzymes was present in sub-optimal amounts.

In the preceding experiments no attempt was made to carry out balance studies or to assess the possible loss of reactants by oxidation processes. In view of the failure to demonstrate the transfer of electrons to oxygen and the

apparent deficiency of respiratory enzyme cofactors in the extracts it was felt that loss of reactants by oxidation processes would be of only minor importance.

The relatively high level of glutamate-aspartate transamination is at variance with the observations on a number of other systems (40, 61, 80). However, Cammarata and Cohen have shown that with pig kidney extracts the glutamate-aspartate system is about 6 times as active as the reaction in which alanine supplies the NH_2 group (16). Neisseria gonorrhoeae has been reported to have an active glutamic-aspartic transaminase but no alanine-glutamic enzyme (98). It has also been noted that the extraction procedures employed may liberate only a portion of the transaminase activity of the original tissue (17). It is quite possible that superior disruption and extraction procedures would indicate a similar situation for the streptomycetes.

Several attempts were made to demonstrate glutamate formation from NH_4^+ and α-ketoglutarate, but these were not successful. This is perhaps not surprising in view of the requirement of glutamic dehydrogenase for DPN previously discussed. Cell extracts were also unable to bring about aspartate synthesis from fumarate and NH_4 ions. During growth in peptonized milk it was found that the medium contained increasing amounts of NH_3 as growth progressed. It may be that under the cultural conditions employed the cells do not develop any appreciable capacity for NH_3 utilization.

The "contamination" of extracts with glutamate and high endogenous glutamate formation have been observed with other tissues (4, 16, 39, 95). To avoid these difficulties it has been suggested that enzyme preparations be dialyzed, concentrated by lyophilization, and excess pyridoxal phosphate added to the reaction mixtures (16). It is possible that a combination of these techniques plus a more efficient disruption procedure would indicate more extensive transaminating activity than has been observed in the present work.

SUMMARY

Techniques were described for the preparation of homogenates of Streptomyces scabies with assured respiratory activity. Data were presented to show that exogenous oxidation could be consistently demonstrated when young cultures established with large inocula were used. A short starvation period was recommended to reduce endogenous activity. Such homogenates were able to oxidize a number of metabolically important organic acids and amino acids. The data indicated that both types of compounds represented important energy sources for the organisms.

Pronounced lag periods were consistently observed during much of the respiration work. Data obtained with cell extracts indicated that for a number of the substrates (succinate, fumarate, citrate, and glutamate) these lags were probably the result of low cell permeability.

The oxidations of glucose, succinate, and glutamate were incomplete, and observations were described which indicated that considerable assimilation probably occurred. A method was described employing dinitrophenol by which a decrease in assimilation during glucose oxidation could be observed.

On the basis of the observed respiratory quotient and the liberation of soluble nitrogen during endogenous respiration it was concluded that the latter was supported, at least in part, by nitrogenous reserves. Cell extracts were shown

to contain considerable quantities of readily extractable amino acids, particularly glutamic acid.

Malonate inhibition of endogenous, succinate, and glutamate oxidation by cell homogenates was demonstrated. Observations supporting a pathway of glutamate oxidation through α-ketoglutarate and succinate were discussed.

Observations on the oxidation of tyrosine by cell homogenates indicated that the oxidation probably proceeded through dihydroxyphenylalanine to melanin in pigment-forming strains. No evidence was obtained for a preliminary transamination with α-ketoglutarate as is observed in some animal tissues.

Cell extracts prepared from mycelium ground with alumina were unable to cause appreciable O_2 uptake in manometric experiments. A technique was described using the dye 2,6-dichlorophenol indophenol by which dehydrogenase activity could be demonstrated with a number of substrates. Extracts were deficient in pyridine nucleotide as evidenced by the stimulation of dye reduction caused by DPN in the presence of glutamate and malate.

Paper chromatographic analyses revealed that cell homogenates and extracts possessed potent glutamic-aspartic transaminase activity. A small but significant glutamate formation was also observed when cell extracts were incubated with α-ketoglutarate and alanine, valine, or leucine. Negligible activity was observed with threonine, lysine,

glycine, and tyrosine as NH_2-donors. No conclusive evidence for direct transamination between α-ketoglutarate and the amides, asparagine, and glutamine could be obtained. Difficulties encountered in the demonstration of transaminase activity were discussed.

REFERENCES

1. Afanasiev, M.M. Comparative physiology of Actinomyces in relation to potato scab. Nebraska Agric. Expt. Sta. Bull. 92: 1-63, 1937.

2. Allen, P.J. Toxins and tissue respiration. Phytopath. 43: 221-229, 1953.

3. Atkinson, D.E. The biochemistry of Hydrogenomonas. II. The adaptive oxidation of organic substrates. J. Bact. 69: 310-315, 1955.

4. Awapara, J. and B. Seale. Distribution of transaminases in rat organs. J. Biol. Chem. 194: 497-502, 1952.

5. Back, K.J.C. and R. Mitchell. The effect of glucose in the growth medium on the succinic acid oxidizing system of Escherichia coli. Aust. J. Sci. Res. (B) 5: 256-263, 1952.

6. Barrett, J.T. and R.E. Kallio. Terminal respiration in Pseudomonas fluorescens: component enzymes of the tricarboxylic acid cycle. J. Bact. 66: 517-525, 1953.

7. Beevers, H. 2,4-dinitrophenol and plant respiration. Amer. J. Bot. 40: 91-96, 1953.

8. Benedict, R.G., Pridham, T.G., Lindenfelser, L.A., Hall, H.H. and R. W. Jackson. Further studies in the evaluation of carbohydrate utilization tests as aids in the differentiation of species of Streptomyces. App. Microbiol. 3: 1-6, 1955.

9. Bennett, R.E. Nutrition of Streptomyces griseus in relation to streptomycin production. J. Bact. 53: 254, 1947. (Abst.)

10. Bentley, M.L. Enzymes of pathogenic fungi. J. Gen. Microbiol. 8: 365-377, 1953.

11. Bernheim, F. and W.E. De Turk. The effect of chloramphenicol and certain other drugs on the oxidation of aromatic amino acids by a strain of Pseudomonas aeruginosa. J. Pharm. and Expt. Therap. 105: 246-251, 1952.

12. Block, R.J., Le Strange, R. and G. Zweig. Paper Chromatography. A Laboratory Manual. Academic Press Inc., N.Y. 1952.

13. Blumenthal, H.J., Koffler, H. and E.P. Goldschmidt. The rate of endogenous respiration as affected by the oxidation of exogenous substrates. Sci. 116: 475-477, 1952.

14. Brodie, A.F. and J.S. Gots. Effects of an isolated dehydrogenase enzyme and flavoprotein on the reduction of triphenyltetrazolium chloride. Sci. 114: 40-41, 1951.

15. Butterworth, E.M., Gilmour, C.M. and C.H. Wang. Studies on the biochemistry of the Streptomyces. II. Fixation of $C^{14}O_2$ by intact cells of Streptomyces griseus. J. Bact. 69: 725-727, 1955.

16. Cammarata, P.S. and P.P. Cohen. The scope of the transamination reaction in animal tissues. J. Biol. Chem. 187: 439-452, 1950.

17. Cammarata, P.S. and P.P. Cohen. Spectrophotometric measurement of transamination reactions. J. Biol. Chem. 193: 45-52, 1951.

18. Campbell, W.R. and M.I. Hanna. The determination of nitrogen by modified Kjeldahl methods. J. Biol. Chem. 119: 1-7, 1937.

19. Cantino, E.C. and M.T. Hyatt. Further evidence for the role of the tricarboxylic acid cycle in morphogensis in Blastocladiella emersonii. J. Bact. 66: 712-720, 1953.

20. Casida, L.E. Jr. and S.G. Knight. The oxidation of tricarboxylic acid cycle compounds by Penicillium chrysogenum. J. Bact. 67: 658-661, 1954.

21. Cavallini, D. and N. Frontali. Quantitative determination of keto acids by paper partition chromatography. Bioch. and Biophy. Acta. 13: 439-445, 1954.

22. Cole, J.O. and C.R. Parks. Semimicro-Kjeldahl procedure for control laboratories. Ind. and Eng. Chem. Anal. Ed. 18: 61-62, 1946.

23. Cochrane, V.W. and I. Dimmick. The metabolism of species of Streptomyces. I. The formation of succinic acid and other acids. J. Bact. 58: 723-730, 1949.

24. Cochrane, V.W. and Jean E. Conn. The metabolism of species of Streptomyces. II. The nitrate metabolism of S. coelicolor. Bull. Torrey Bot. Club 77: 10-18, 1950.

25. Cochrane, V.W. and M. Gibbs. The metabolism of species of Streptomyces. IV. The effect of substrate on the endogenous respiration of Streptomyces coelicolor. J. Bact. 61: 305-307, 1951.

26. Cochrane, V.W. The metabolism of species of Streptomyces. V. The role and pathway of synthesis of organic acids in Streptomyces coelicolor. J. Bact. 63: 459-471, 1952.

27. Cochrane, V.W. and H.D. Peck. The respiratory mechanism of S. scabies. Phytopath. 42: 5, 1952. (Abst.)

28. Cochrane, V.W. and H.D. Peck. The metabolism of species of Streptomyces. VI. Tricarboxylic acid cycle reactions in Streptomyces coelicolor. J. Bact. 65: 37-44, 1953.

29. Cochrane, V.W., Peck, H.D. and Anne Harrison. The metabolism of species of Streptomyces. VII. The hexose monophosphate shunt and associated reactions. J. Bact. 66: 17-23, 1953.

30. Coty, V.F., Garner, H.R. and H. Koffler. Tricarboxylic acid cycle reactions in Streptomyces griseus. Bact. Proc. 1953: 74.

31. Darby, R.T. and D.R. Goddard. Studies on the respiration of the mycelium of the fungus Myrothecium verrucaria. Amer. J. Bot. 37: 379-387, 1950.

32. Denison, F.W. Jr. and E.F. Phares. Rapid method for paper chromatography of organic acids. Anal. Chem. 24: 1628-1629, 1952.

33. Dickens, F. and H. McIlwain. Phenazine compounds as carriers in the hexose monophosphate system. Biochem. J. 32: 1615-1625, 1938.

34. Douglas, R.J. and E.H. Garrard. Serological observations on the actinomycetes associated with potato scab. Can. J. Bot. 32: 480-485, 1954.

35. Eimhjellen, K.E. and H. Larsen. The mechanism of itaconic acid formation by Aspergillus terreus. 2. The effect of substrates and inhibitors. Biochem. J. 60: 139-147, 1955.

36. Emerson, S. The growth phase in Neurospora corresponding to the logarithmic phase in unicellular organisms. J. Bact. 60: 221-223, 1950.

37. Englesberg, E. and J.B. Levy. Induced synthesis of tricarboxylic acid cycle enzymes as correlated with the oxidation of acetate and glucose by Pasteurella pestis. J. Bact. 69: 418-431, 1955.

38. Erikson, D. and D. Webley. The respiration of a thermophilic actinomycete Micromonospora vulgaris. J. Gen. Microbiol. 8: 455-463, 1953.

39. Feldman, L.I. and I.C. Gunsalus. The occurrence of a wide variety of transaminases in bacteria. J. Biol. Chem. 187: 821-830, 1950.

40. Fincham, J.R.S. Effects of a gene mutation in Neurospora crassa relating to glutamic dehydrogenase formation. J. Gen. Microbiol. 11: 236-246, 1954.

41. Fowden, L. and J. Done. A new transamination reaction. Nat. 171: 1068, 1953.

42. Fowden, L. The quantitative recovery and colorimetric estimation of amino acids separated by chromatography. Biochem. J. 48: 327-333, 1951.

43. Garner, H.R. and H. Koffler. Preliminary evidence against the existence of Kreb's cycle in Streptomyces griseus. Bact. Proc. 1951: 139.

44. Gentile, A.C. Carbohydrate metabolism and oxalic acid synthesis by Botrytis cinerea. Plant Physiol. 29: 257-261, 1954.

45. Gerhardt, P., MacGregor, D.R., Marr, A.G., Olsen, C.B. and J.B. Wilson. The metabolism of Brucella: The role of cellular permeability. J. Bact. 65: 581-586, 1953.

46. Gilmour, C.M., Butterworth, E.M., Noble, E.P. and C.H. Wang. Studies on the biochemistry of the Streptomyces. I. Terminal oxidative metabolism in Streptomyces griseus. J. Bact. 69: 719-724, 1955.

47. Gordon, R.E. and M.M. Smith. Proposed group of characters for the separation of Streptomyces and Nocardia. J. Bact. 69: 147-150, 1955.

48. Gothoskar, S.S., Scheffer, R.P., Stahmann, M.A. and J.C. Walker. Further studies on the nature of Fusarium resistance in tomato. Phytopath. 45: 303-307, 1955.

49. Gottlieb, D. The physiology of the actinomycetes. Symposium (Actinomycetales) VI. Int. Cong. Microbiol. Rome, 1953.

50. Gottlieb, D. and H.W. Anderson. The respiration of Streptomyces griseus. Sci. 107: 172-173, 1948.

51. Goucher, C.R., Woodside, E.E. and W. Kocholaty. The influence of 2,4-dinitrophenol on the oxidation of acetate and succinate by Escherichia coli. J. Bact. 67: 593-596, 1954.

52. Green, M. and P.W. Wilson. Hydrogenase and nitrogenase in Azotobacter. J. Bact. 65: 511-517, 1953.

53. Haas, E. A colorimetric determination for studies involving coenzymes. J. Biol. Chem. 155: 333-335, 1944.

54. Harris, J.O. A study of adaptation in Azotobacter. Soil Sci. Soc. Amer. Proc. 18: 154-156, 1954.

55. Hockenhull, D.J.D., Walker, A.D., Wilkin, G.D. and F.G. Winder. Oxidation of phenylacetic acid by Penicillium chrysogenum. Biochem. J. 50: 605-609, 1952.

56. Hollis, J.P. Studies on Streptomyces scabies. II. Stimulation of melanin production by amino acids. Bull. Torrey Bot. Club. 81: 98-103, 1954.

57. Horowitz, N.H. and S.C. Shen. Neurospora tyrosinase. J. Biol. Chem. 197: 513-520, 1952.

58. Hugo, W.B. The preparation of cell-free enzymes from microorganisms. Bact. Revs. 18: 87-105, 1954.

59. Isenberg, H.D., Schatz, A., Angrist, A.A., Schatz, V. and G.S. Trelawny. Microbial metabolism of carbamates. II. Nitrification of urethan by Streptomyces nitrificans. J. Bact. 68: 5-9, 1954.

60. Jones, N.R. The free amino acids of fish. 1-methyl histidine and β-alanine liberation by skeletal muscle anserinase of codling (Gadus callarias). Bicchem. J. 60: 81-87, 1955.

61. Jordan, D.C. Transamination in cell-free extracts of effective and paratistic Rhizobia. J. Bact. 65: 220-221, 1953.

62. Kann, E.E. and R.C. Mills. Oxidation of glutamic acid by Pasteurella tularensis. J. Bact. 69: 659-664, 1955.

63. Koffler, H. (convenor) Symposium on physiology of fungi. Cornell University, Ithaca, 1952.

64. Koffler, H. Problems of oxidative mold metabolism. Symposium (Microbial Metabolism) VI. Int. Cong. Microbiol. Rome, 1953.

65. Lerner, A.B. and T.B. Fitzpatrick. Biochemistry of melanin formation. Physiol. Revs. 30: 91-126, 1950.

66. Lerner, A.B. Metabolism of phenylalanine and tyrosine. Adv. in Enz. 14: 73-128, 1953.

67. Lindstrom, E.S. The α-ketoglutarate oxidase system of Azotobacter. J. Bact. 65: 565-570, 1953.

68. Marr, A.G., Olsen, C.B., Unger, H.S. and J.B. Wilson. The oxidation of glutamic acid by Brucella abortus. J. Bact. 66: 606-610, 1953.

69. Meister, A. in Amino Acid Metabolism. ed. by W. D. McElroy and B. Glass. John Hopkins Press, 1955.

70. Meister, A. Transamination. Adv. in Enz. 16: 185-246, 1955.

71. Millman, I. and G.P. Youmans. The characterization of the terminal respiratory enzymes of the H37Ra strain of Mycobacterium tuberculosis. J. Bact. 69: 320-325, 1955.

72. Mohan, R.R., Trelawny, G.S. and A. Schatz. Microbial metabolism of carbamates. III. Preparation and respiratory activity of nonproliferating cells of Streptomyces nitrificans. J. Bact. 69: 387-392, 1955.

73. Neish, A.C. Analytical methods for bacterial fermentations. Nat. Res. Council of Can. Report 46-8-3 R, 1950.

74. Newbould, F.H.S. and E.H. Garrard. Studies on actinophage for Streptomyces scabies. (Thaxt.) Waksman and Henrici. Can. J. Bot. 32: 386-391, 1954.

75. Nickerson, W.J. and R.R. Mohan. Studies on the nutrition and metabolism of Streptomyces. Symposium (Actinomycetales) VI. Int. Cong. Microbiol. Rome, 1953.

76. Nickerson, W.J. and F.G. Sherman. Metabolic aspects of bacterial growth in the absence of cell division. II. Respiration of normal and filamentous cells of Bacillus cereus. J. Bact. 64: 667-678, 1952.

77. Oginsky, E.L., Smith, P.H. and W.W. Umbreit. The action of streptomycin on the respiration of Streptomyces griseus. J. Bact. 61: 639-642, 1951.

78. Perlman, D. and G. Wagman. Studies on the utilization of lipids by Streptomyces griseus. J. Bact. 63: 253-262, 1952.

79. Perlman, D. Physiological studies on the actinomycetes. Bot. Rev. 19: 46-97, 1953.

80. Pollak, J.K. and D. Fairbairn. The metabolism of Ascaris lumbricoides ovaries. II. Amino acid metabolism. Can. J. Biochem. and Physiol. 33: 307-316, 1955.

81. Price, C.A. and K.V. Thimann. The estimation of dehydrogenases in plant tissue. Plant Physiol. 29: 113-124, 1954.

82. Repaske, R. and P.W. Wilson. Oxidation of intermediates of the tricarboxylic acid cycle by extracts of Azotobacter agile. Proc. Nat. Acad. Sci. 39: 225-232, 1953.

83. Richardson, J.K. The influence of tuber development on scab infection in Katahdin potatoes. Phytopath. 42: 297-298, 1952.

84. Rolinson, G.N. Respiration of Penicillium chrysogenum in penicillin fermentations. J. Gen. Microbiol. 6: 336-343, 1952.

85. Rowsell, E.V. Transamination to pyruvate and some other α-keto acids. Nat. 168: 104, 1951.

86. Schaal, L.A. Variation and physiological specialization in the common scab fungus. (A. scabies.) J. Agric. Res. 69: 169-186, 1944.

87. Schepartz, B. Transamination as a step in tyrosine metabolism. J. Biol. Chem. 193: 293-298, 1951.

88. Simon, E.W. The action of nitrophenols on respiration and on glucose assimilation in yeast. J. Expt. Bot. 4: 377-392, 1953.

89. Simon, E.W. and H. Beevers. The quantitative relationship between pH and the activity of weak acids and bases in biological experiments. Sci. 114: 124-126, 1951.

90. Spencer, R.P. and J.B. Field. Qualitative colorimetric assay of tyrosinase substrates and inhibitors. P.S.E.B.M. 88: 576-578, 1955.

91. Stout, H.A. and H. Koffler. Biochemistry of filamentous fungi. I. Oxidative metabolism of glucose by Penicillium chrysogenum. J. Bact. 62: 253-268, 1951.

92. Taylor, C.F. and P. Decker. A correlation between pathogenicity and cultural characteristics in the genus Actinomyces. Phytopath. 37: 49-58, 1947.

93. Terner, C. The metabolism of citric acid in the mammary gland. I. The effect of p-nitrophenol on the synthesis and oxidation of citric acid by homogenates of mammary gland. Biochem. J. 60: 88-95, 1955.

94. Terner, C. The metabolism of citric acid in the mammary gland. II. The effect of p-nitrophenol and of fluoride on the synthesis of citric acid in fluoroacetate-blocked homogenates. Biochem. J. 60: 95-101, 1955.

95. Thorne, C.B., Gomez, C.G. and R.D. Housewright. Transamination of D-amino acids by Bacillus subtilis. J. Bact. 69: 357-362, 1955.

96. Thorne, M.B. Inhibition by malonate of succinic dehydrogenase in heart-muscle preparations. Biochem. J. 54: 540-547, 1953.

97. Tissieres, A., Mitchell, H.K. and F.A. Haskins. Studies on the respiratory system of the poky strain of Neurospora. J. Biol. Chem. 205: 423-433, 1953.

98. Tonhazy, N.E. and M.J. Pelczar Jr. Oxidation of amino acids and compounds associated with the tricarboxylic acid cycle by Neisseria gonorrhoeae. J. Bact. 65: 368-377, 1953.

99. Umbarger, H.E. The influence of the environment on acetate metabolism in Escherichia coli. J. Bact. 68: 140-145, 1954.

100. Umbreit, W.W. Respiratory cycles. J. Cell. and Comp. Physiol. 41 (Supp. I): 39-66, 1953.

101. Umbreit, W.W., Burris, R.H. and J.F. Stauffer. Manometric techniques and tissue metabolism. Burgess Pub. Co., Minn. 1949.

102. Vaisey, E.B. An investigation of actinomycetes isolated from the soil and their ability to cause potato scab. M.S.A. Thesis, University of Toronto, 1953.

103. Waksman, S.A. Neomycin. Rutgers University Press, 1953.

104. Webley, D.M. The effect of oxygen on growth and metabolism of the aerobic thermophilic actinomycete Micromonospora vulgaris. J. Gen. Microbiol. 11: 114-122, 1954.

105. Williams, A.M. and P.W. Wilson. Equilibration of succinate solutions with adapted and unadapted Azotobacter cells. Can. J. Microbiol. 1: 36-44, 1954.

106. Williams, A.M. and P.W. Wilson. Adaptation of Azotobacter cells to tricarboxylic acid substrates. J. Bact. 67: 353-360, 1954.

107. Wilner, B. and C.E. Clifton. Oxidative assimilation by Bacillus subtilis. J. Bact. 67: 571-575, 1954.