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LEUCINE IN CULTURED CELLS: ITS METABOLISM AND USE AS A MARKER FOR PROTEIN TURNOVER

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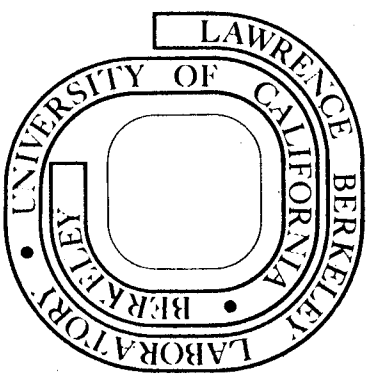

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LEUCINE IN CULTURED CELLS: ITS METABOLISM AND USE AS A MARKER FOR PROTEIN TURNOVER

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Running Title: LEUCINE METABOLISM IN CULTURED CELLS

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SUMMARY

The metabolism of leucine by normal and virally transformed mouse fibroblasts in culture has been investigated. Cultures pulsed with (U)^{14}C-leucine were studied for the distribution of radioactivity in the free amino acid pool, cellular protein, lipid and CO_2. Less than 1% of the label taken up was found in lipid and no radioactivity was recovered as CO_2 when cultures were pulsed in growth medium. That these cells retain the capacity to oxidize leucine was shown by experiments in which cultures pulsed in a balanced salt solution (Krebs-Ringer bicarbonate) showed linear evolution of CO_2.

Leucine proved to be a desirable marker for protein turnover studies due to its minimal metabolism when labeling was performed in growth medium. The appearance of radioactive leucine in the medium of cells previously labeled with ^{14}C-leucine was shown to be directly correlated with loss of radioactivity in cellular protein. A class of rapidly degrading proteins and a second group of proteins with a slower rate of turnover could be distinguished when cells were labeled with ^{14}C- and ^{3}H- leucine for varying lengths of time. Density inhibited cultures degraded both these classes of proteins about twice as rapidly as did growing cells.

Alterations in rates of protein synthesis, especially for specific classes of proteins (e.g., non-histone chromosomal proteins) have been implicated in recent years as one of the processes which may contribute to the regulation of growth of cells in culture (1-3). Since proteins are continuously turning over (4), the degradation of proteins must also be involved in any such control mechanism. While the control of protein synthesis has been the subject of numerous investigations, very little is known about protein degradation in tissue culture cells.

Analysis of protein turnover using radioactively labeled proteins is complicated by degradation of the labeled amino acid itself. It is therefore desirable to use an amino acid which is not synthesized by the cells under study and which is, in addition, minimally metabolized. Isotopically labeled leucine has been frequently employed in protein turnover studies (5-8). Previous investigations have used the appearance of radioactive leucine in the growth medium as an assay for intracellular protein degradation (5). This is a convenient method and permits detection of both rapid and small changes in degradative rates which might not be observable in assays which account for degradation by loss of label from a high background of radioactive proteins. However, it has been shown in muscle tissue and in human fibroblasts in culture that there is considerable oxidation of leucine to CO_2 (9-11). Such extensive oxidation would invalidate the measurement of free leucine in growth medium as an assay of intracellular protein degradation. We have, therefore, investigated leucine metabolism in non-transformed and MSV/MLV transformed Balb 3T3 A31 fibroblasts

in order to determine the extent and nature of leucine degradation in culturedcells. We show that while oxidative degradation of leucine by cells pulsed in growth medium is minimal, evolution of CO_2 from leucine is highly dependent upon the complexity of the medium in which such experiments are carried out.

Using the appearance of leucine in the medium, Poole and Wibo have recently demonstrated the existence of proteins with long and short half-lives in confluent populations of rat fibroblasts (5). In work reported here we have compared the rate of turnover of these two classes of proteins in actively growing and in non-cycling density-inhibited Balb 3T3 fibroblasts. The results indicate that growing cells degrade both classes of proteins more slowly than do quiescent, density-inhibited cells.

MATERIALS AND METHODS

Cell Culture

A cloned line of Balb 3T3 A31 mouse fibroblasts originally obtained from the Naval Biomedical Laboratory, Oakland, California, and a line of Balb 3T3 A31 cells transformed by the Moloney strains of murine sarcoma and leukemia virus (MSV/MLV Balb 3T3 A31) were cultured in 35 mm Falcon plastic petri dishes in Dulbecco's Modified Eagles Medium (DME)(Gibco H-16) supplemented with 10% donor calf serum (Flow Laboratories). Cells were seeded two days prior to experimental use at either high (2×10^5 cells/dish) or low (2×10^4 cells/dish) density. Cells for each experiment were tested for the presence of mycoplasma by ^{3}H-thymidine autoradiography (12), and were found

to be uncontaminated. For each experiment, duplicate plates were labeled with ^{3}H-thymidine (1 μCi/ml, New England Nuclear) for six hours, and the percentage of labeled nuclei was detemined. Density-inhibited cells had fewer than 1.0% labeled nuclei and growing cells showed 45 to 50% of the nuclei to be labeled.

Isotope

L-[4, 5-^{3}H]-leucine (5Ci/mmole) and 1-[^{14}C(U)]-leucine (299mCi/mmole) were obtained from New England Nuclear. Although the ^{14}C-leucine was found to be pure by the two-dimensional paper chromatography and autoradiography system used in this laboratory (13), we have consistently collected a volatile impurity which accounts for 0.2 to 0.3% of the total radioactivity. This led to a rather high background level of radioactivity which could not be eliminated and which, given the sensitivity of the CO_2 absorption system used, could obscure minimal oxidation of ^{14}C-leucine.

Labeling of Cells

Depending upon the experiment, cells were labeled in DME containing the normal amount of dialyzed serum (10%) or in Krebs-Ringer bicarbonate buffer (14) containing 0.4% dialyzed donor calf serum.

For turnover experiments, monolayers were exposed to medium containing ^{3}H- or ^{14}C-leucine for varying lengths of time (as discussed in the results). At the end of the labeling period, radioactive medium was removed and cell monolayers washed 3 times with warm Earles Balanced Salt Solution.(BSS) Unlabeled medium (conditioned medium) from cultures seeded at the same time and densities as experimental cultures was then pooled and adjusted to contain cold leucine at a concentration of 10 mM. This medium was then added to the prelabeled cultures at zero time and at various times thereafter

radioactivity in the medium, free amino acid pool, and protein was assayed. Amino acid analyses (Beckman Model 120 C Amino Acid Analyzer) were performed on conditioned medium used for labeling in order to determine specific activities. For metabolic studies cell monolayers were washed 3 times in warm Earles Balanced Salt solution and ^{14}C-leucine was added at equal specific activities in either DME or Krebs-Ringer buffer with the addition of 0.4% dialyzed calf serum.

Extraction and Analyses of Free Amino Acid Pool and Protein

Trichloracetic acid (TCA) soluble radioactivity was extracted by overlaying washed cell monolayers with 1 ml of cold 5% TCA for 10 minutes. The TCA was then removed, and the monolayer washed 3 times with cold PBS. The TCA insoluble material remaining on the plate was then dissolved in .1% SDS-.01N NaOH. Aliquots of medium were counted both in Aquasol II to give total radioactivity, and precipitated with 10% TCA, filtered onto Whatman GFC filter paper and counted in a Toluene POPOP cocktail to yield TCA precipitable radioactivity (regularly less than 4% of the total counts). Counts were corrected for quench, background and spillover (where applicable) and converted to DPM. Protein concentrations were determined by the method of Lowry (15) using bovine serum albumin as a standard. For metabolic studies, the small amount of radioactivity in cellular lipid was subtracted from the total radioactivity in TCA precipitable material to yield corrected values for radioactively labeled protein.

Extraction and Analysis of Cellular Lipid

At the end of the labeling period, cell monolayers were washed 3 times with cold PBS. For lipid extraction, cells were grown in 60 mm glass petri dishes. Total lipid was extracted from cell monolayers by a modification of the procedure of Folch, et al. (16). A 2 ml amount of chloroform-methanol

(2:1, V/V) was added immediately to each washed plate and allowed to remain at room temperature for 20 minutes. The solvent was then transferred to a centrifuge tube and each plate washed with an additional 2 ml of chloroform-methanol and volumes were adjusted to 4 ml. Each tube then received 0.5 ml of 0.05 M NaCl and the tubes were shaken, allowed to stand and shaken again. The chloroform layer was withdrawn by a Pasteur pipette inserted under the aqueous phase. NaCl (0.5 ml of .05 M) was again added to the chloroform layer and the extraction was repeated a second time. The chloroform layer was transferred to a scintillation vial and allowed to evaporate to dryness. Scintillation fluid (toluene POPOP) was added and samples counted as described above.

Collection of CO_2

A modified procedure of Bissell, et al. (17) was employed. Petri dishes (35 mm) containing 1 ml of radioactive medium were placed into 100 ml beakers and gassed with 10% CO_2 and 90% air for 30 seconds. Beakers were then plugged with 1-hole rubber stoppers into which serum stoppers had been inserted. Cells during the labeling period were maintained at 37°C. At the end of this period, 0.2 ml 2 N H_2SO_4 were injected by syringe into each petri dish. NCS (Packard) was then injected in 0.2 ml aliquots into a small plastic cup previously inserted through the serum stopper and CO_2 was absorbed for 90 minutes in the stoppered beakers. The plastic cup was then removed, placed in a scintillation vial containing toluene POPOP and counted. The efficiency of CO_2 evolved and collected was tested with a known amount of ^{14}C-$NaHCO_3$.

Two dimensional chromatography and autoradiography

Cell monolayers were labeled for one hour with $^{14}C(U)$ l-leucine (15 uCi/ml) in growth medium (DME) + 0.4% dialyzed calf serum. Cells were washed 3 times with cold phosphate buffered saline and killed with 1 ml 80% methanol. They were then removed by scraping and the volume of methanol reduced under nitrogen. Chromatograms were spotted, run, and exposed to X-ray film; films were developed and spots quantified as described by Bassham, et al.(13).

RESULTS

Leucine metabolism in cultured cells

Cells labeled with $^{14}C(U)$-leucine were analyzed by two dimensional chromatography and autoradiography. These studies revealed no labeled metabolic intermediates other than a small pool of acetoacetate. In order to determine the distribution of utilized leucine, labeled cells were next assayed for radioactivity in the free amino acid pool, protein, cellular lipid and CO_2. The rate of protein synthesis, as expected, was linear for at least 90 minutes and was directly proportional to the rate of growth - with non-transformed actively growing, and transformed cells showing higher rates of protein synthesis than the non-cycling density-inhibited cells (fig. 1A). In all cases the TCA soluble pool of radioactivity reached steady-state equilibrium values by 5 minutes after addition of the label (data not shown). The rate of lipid biosynthesis from leucine is shown in figure 1B. Incorporation of leucine into lipid was linear for at least 30 minutes and was inversely correlated with the rate of growth of the cells; non-transformed density-inhibited cells showed the highest rate of

lipid synthesis and transformed cells showed the lowest. A decreased rate of lipid synthesis may reflect an actual shift in metabolic activity of these cells in response to an altered growth rate. For purposes of this study, however, these results indicate that a negligible amount of radioactivity (1% of the total, Table I) is found in cellular lipid (however, see discussion).

Contrary to observations on rat diaphragm in vitro and human fibroblasts in culture (9-11), no CO_2 evolution could be detected from ^{14}C-leucine in intial experiments. Since the reported data of in vitro leucine oxidation was obtained from tissue or cells pulsed in a balanced salt buffer (Krebs bicarbonate)(9-11, 18), cell monolayers were pulsed with $^{14}C(U)$ l-leucine in Krebs-Ringer buffer. While Balb 3T3 A31 fibroblast pulsed in growth medium did not convert leucine to CO_2, cells labeled in buffer oxidized leucine at a linear rate (fig. 2). To test whether the inhibitory effect on leucine oxidation in the medium was due to the presence of glucose and sodium pyruvate or amino acids, CO_2 evolution in buffer was measured after addition of these compounds. It was found that CO_2 production decreased by about 60% when glucose and pyruvate were added to Krebs-Ringer buffer at concentrations found in DME (fig. 2). If the complete amino acid mixture found in DME were added to the buffer, no CO_2 evolution was observed (fig. 2). These experiments indicate that mouse fibroblasts in culture retain the capacity to oxidize leucine, but that in growth medium this is not a major metabolic pathway of leucine degradation.

In the course of our investigation of CO_2 evolution in Krebs-Ringer buffer, fibroblasts pulsed with ^{14}C l-leucine in Krebs-Ringer solution showed a rate of protein synthesis which was about 4 times as great as the rate observed in cells in growth medium (DME)(fig. 3). Since there

were no other amino acids present for protein synthesis in the buffer, and since the total amount of protein did not increase over values obtained for cells in DME, these results indicate that protein turnover was accelerated by cells in Krebs-Ringer buffer. The radioactive free leucine pool in cells pulsed in buffer was approximately twice as large as that of cells in DME. However, this increase in pool radioactivity was not great enough to account for the increased rate of synthesis observed.

The results of experiments carried out in growth medium show that leucine is not extensively metabolized by mouse fibroblasts in culture. Table I summarizes these data. The metabolic regulation of leucine by mouse cells in DME is such that leucine, while continuing to contribute to protein synthesis and to a minimal degree, lipid synthesis, is not oxidized to CO_2. This metabolic pattern can be altered by placing cells in another extracellular environment, as has been shown with experiments carried out in Krebs-Ringer buffer.

The rate of protein degradation in growing and quiescent cells

Having established that there is minimal metabolism of leucine by cultured cells in growth medium, the average rate of protein turnover in growing and quiescent Balb 3T3 A31 cells was examined. After removal of radioactive medium from cells labeled for 56 hours with ^{14}C-leucine, the disappearance of radioactivity from protein and its appearance in the medium were monitored. In agreement with our previous results, TCA soluble radioactivity in the medium was directly proportional to loss of radioactivity in protein (fig. 4). Two dimensional chromatography and autoradiography

of the medium (see Materials and Methods) identified the only labeled compound in the medium as leucine. In addition it was found that the intracellular free leucine pool equilibrated rapidly with the medium (fig. 5). By five minutes after the removal of medium containing ^{14}C-leucine and the addition of fresh medium containing cold leucine, approximately 90% of the label originally present in the intracellular free amino acid pool appeared in the medium. In order to prevent any reutilization of this labeled leucine and also to establish a lower background for determining radioactive leucine derived from protein breakdown, labeled cultures were flushed for five minutes with unlableled conditioned medium in protein degradation experiments. This medium was then discarded and unlabeled conditioned medium containing 10 mm cold leucine again added. Samples of this medium were subsequently assayed for radioactivity to determine the rate of average protein degradation.

In order to examine degradative rates of total cellular proteins with long and short half lives, cells were labeled with ^{3}H- and ^{14}C-leucine for varying lengths of time. Actively growing and quiescent, density-inhibited cells were studied in these experiments to first determine protein turnover rates which are correlated with rate of growth. Cells were labeled for 24 hours with ^{14}C-leucine and 1 hr with ^{3}H-leucine as described in the legend of figure 6. The rate of appearance of ^{3}H-leucine was more rapid with respect to the total amount of ^{3}H-leucine label in the cells at the time of label removal than the rate of appearance of ^{14}C-leucine in both growing and quiescent cells (fig. 6). This is indicated by the initial rapid appearance of ^{3}H-leucine in the medium followed by a slower rate as the supply of labeled rapidly turning over protein is diminished. Cells which have ceased growing (as judged by incorporation of ^{3}H-thymidine) and autoradiography

degraded both these classes of protein about twice as rapidly as did actively growing mouse fibroblasts (fig. 6).

DISCUSSION

Oxidation in non-hepatic tissue is thought to be a major pathway of degradation of leucine, valine and isoleucene (9,10,19). In excised rat diaphragm, Odessey and Goldberg found that 38% of the labeled leucine taken up could be recovered as CO_2 (9). Human fibroblasts in culture were also shown to oxidize leucine (11). These data, however, were obtained from cells or tissue pulsed in a simple salt buffer (Krebs-Ringer) which is frequently used in physiological experiments. When glucose or amino acids at concentrations found in plasma were added, oxidation of leucine was decreased (9). Tissue culture growth medium has been developed to yield optimum growth of cells in culture. While no tissue culture environment (buffer or even growth medium) can accurately mimic *in vivo* conditions, we found that Balb 3T3 A31 cells will not survive longer than three hours in Krebs-Ringer buffer even in the presence of 0.4% serum. Our results showing accelerated protein turnover in Krebs-Ringer buffer and the absence of leucine oxidation by cells in growth medium demonstrate the ability of cells to alter their metabolism according to the extracellular environment. Wettenhall, *et al.* (20), have reported a difference in the rate of protein synthesis by lymphocytes in Krebs-Ringer buffer versus growth medium. In addition we have found that metabolite patterns derived

from ^{14}C-glucose labeled chick embryo fibroblasts are strikingly different when cells are pulsed in buffer (Hanks Balanced salt solution or Krebs) in place of growth medium. (Bissell and Neff, unpublished). These results demonstrate the importance of identifying as carfully as possible those metabolic processes which may vary under different experimental conditions, and in correlating their relevance to conditions in vivo.

Although less than 1% of the total label taken up was recovered in cellular lipid, our results indicated that the rate of lipid biosynthesis from leucine increased as cellular growth rate decreased. However, this data should be interpreted with reservation since accurate determination of rates of lipid biosynthesis by cells in culture is difficult due to their acquisition of lipids from serum fatty acids (21, 22). Although labeling experiments were carried out in medium containing serum at a low concentration (0.4%), cells in this laboratory are routinely cultivated in medium supplemented with 10% serum. Our data do not take into consideration cellular lipid acquired before the labeling period.

Reutilization of the labeled amino acid causes difficulties in the interpretation of protein turnover experiments. Righetti, et al., report that reutilization of leucine in Hela cells is as high as 81% (23). It is possible that growing and density-inhibited cells may reutilize amino acids to varying degrees. However, the addition of 2, 10, and 20 mM cold leucine to the medium of growing and quiescent cells which had been previously labeled with ^{14}C-leucine, did not change the rate of appearance of radioactivity in the medium for time periods up to 48 hours after label removal. In addition, Poole, et al. (5), have pointed out that cells grown in a monolayer present an enormous surface area for amino acid exchange. The

extracellular concentration of amino acid is very large in comparison to the intracellular pool, so that in monolayer culture systems there may be little reutilization. Even though this may be an accurate assumption, in the absence of direct proof, rates of protein degradation estimated by the isotopic techniques used in this communication represent relative values.

Conditioned medium was used in experiments measuring the rate of protein degradation in order to minimize the possible effects of fresh medium and serum on the rates of protein degradation. Stimulation of DNA synthesis in density-inhibited cells by serum and fresh medium is known to be accompanied by changes in rates of protein synthesis (24). Such alterations in the cellular environment could therefore also be expected to change rates of protein breakdown. Cold leucine was added at high concentrations (10mM) to conditioned medium to prevent experimental artifacts due to differences in cold leucine concentrations. MSV/MLV transformed mouse fibroblasts were also examined in these studies. The medium from these cells exhibited a higher percentage of TCA insoluble radioactivity, presumably due to virus production and a higher percentage of detached cells, and results of experiments varied according to the degree of transformation. However, MSV/MLV transformed mouse fibroblasts showed rates of protein breakdown similar to or slightly higher than actively growing normal fibroblasts.

The increased synthesis of protein in growing cells observed in these studies is accompanied by decreased turnover of proteins with both rapid and slow turnover rates. Density-inhibited cells, on the other hand, show a decreased rate of protein synthesis and a more rapid rate of protein degradation. Protein synthesis and degradation are thus intimately involved

in growth regulation and the maintenance of the density-inhibited state. We are continuing to investigate the degradation of proteins derived from fractionated cells and how their turnover rates may be related to growth regulation in culture.

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Table I

Cell monolayers were pulsed as described in fig. 1(A). TCA soluble and insoluble fractions were assayed and lipid extracted as described in Materials and Methods. Each value represents the mean of 3 separate determinations.

Figure Legends

Fig. 1. Abscissa: Minutes after addition of ^{14}C-leucine; ordinate: DPM/ug protein.

(A) TCA precipitable radioactivity. Cell monolayers were incubated in the presence of (U) ^{14}C-leucine at a final specific activity of 1.23 μCi/umole in DME containing 0.4% dialyzed calf serum.

(B) Radioactivity in total cellular lipid. Cells were labeled as in fig. 1(A) and lipid was extracted as described in Materials and Methods. Non-transformed density-inhibited cells ⊙; non-transformed growing cells o, MSV/MLV-transformed cells Δ. Each point is the mean of 3 separate determinations.

Fig. 2. Abscissa: Minutes after addition of ^{14}C-leucine; ordinate: DPM/ug protein. CO_2 evolution from ^{14}C-leucine. Monolayers were incubated in the presence of (U) ^{14}C-leucine at a specific activity of 1.23 μCi/umole in either DME or Krebs-Ringer bicarbonate buffer both of which were supplemented with 0.4% dialyzed calf serum. CO_2 was collected as described in Materials and Methods. Krebs-Ringer buffer o, Krebs-Ringer buffer + 27 mM glucose and .9 mM Na-pyruvate ⊙, Krebs-Ringer buffer + DME amino acids Δ, DME Δ. Each point is the average of 2 separate determinations.

Fig. 3. Abscissa: Minutes after addition of ^{14}C-leucine; ordinate: DPM/ug protein. TCA precipitable radioactivity. Monolayers were pulsed with ^{14}C-leucine as described in fig. 2. Krebs-Ringer buffer o, DME Δ. Each point is the mean of 3 separate determinations.

Fig. 4. Abscissa: Minutes after removal of label; ordinate: DPM x 10^{-3} in TCA soluble pool. TCA soluble radioactivity in the free amino acid pool and medium after label removal. Cell monolayers (Balb 3T3 A31) were pulsed 1 hour with ^{3}H-leucine (2μCi/ml) in DME containing 10% calf serum. Radioactive medium was removed, monolayers washed and fresh medium added. TCA soluble radioactivity in the pool(●) and medium (o) was assayed. Each point is the mean of 3 separate determinations.

Fig. 5 Abscissa: Hours after removal of label; ordinate: % of total radioactivity in protein (●) and in medium (o). Disappearance of radioactivity from protein versus its appearance in the medium. Density-inhibited Balb 3T3 A31 cells were labeled for 56 hours in DME containing 10% dialyzed calf serum and 1μCi/ml (U)^{14}C-leucine. At the end of the labeling period, monolayers were washed 3 times with Earles Balanced Salt Solution and fresh DME containing 0.4% dialyzed calf serum was added. Radioactivity in the medium, TCA soluble pool and protein was assayed at varying times thereafter. Total radioactivity is the sum of the DPM in the TCA soluble pool, and in protein at the time of label removal. Each point is the mean of 3 determinations.

Fig. 6 Abscissa: Hours after removal of label; ordinate: % of total radioactivity. TCA soluble radioactivity appearing in the medium after label removal, (●) density-inhibited cell, (o) growing cells; A, ^{3}H-leucine labeled cells; B, ^{14}C-leucine labeled cells. Cell monolayers were labeled for 24 hours with 1μCi/ml (U) ^{14}C-leucine in DME. At the end of this period, medium was removed

and the monolayer washed with warm BSS. Conditioned medium containing 10 μCi/ml ^{3}H-leucine was then added to the culture for 1 hour. This medium was then removed and the cultures flushed for five minutes with unlabeled conditioned medium. The flushing medium was removed and replaced with conditioned medium containing 10mM cold leucine. Radioactivity in the medium was determined at various times thereafter. Total radioactivity is the sum of DPM of each isotope in the TCA soluble pool and protein at the time of label removal. Each point is the mean of 12 separate determinations.

TABLE I

Cells	Minutes after addition of ^{14}C-leucine	% Radioactivity in		
		TCA soluble	Protein	Lipid
Non-transformed, Density-Inhibited	15	82.8	16.8	.36
	60	57.5	41.2	1.20
	90	46.4	52.5	.93
Non-transformed, Growing	15	56.0	43.4	.50
	60	26.1	73.0	.80
	90	13.5	85.8	.57
MSV/MLV Transformed	15	44.5	55.2	.20
	60	19.6	80.0	.28
	90	12.9	86.8	.26

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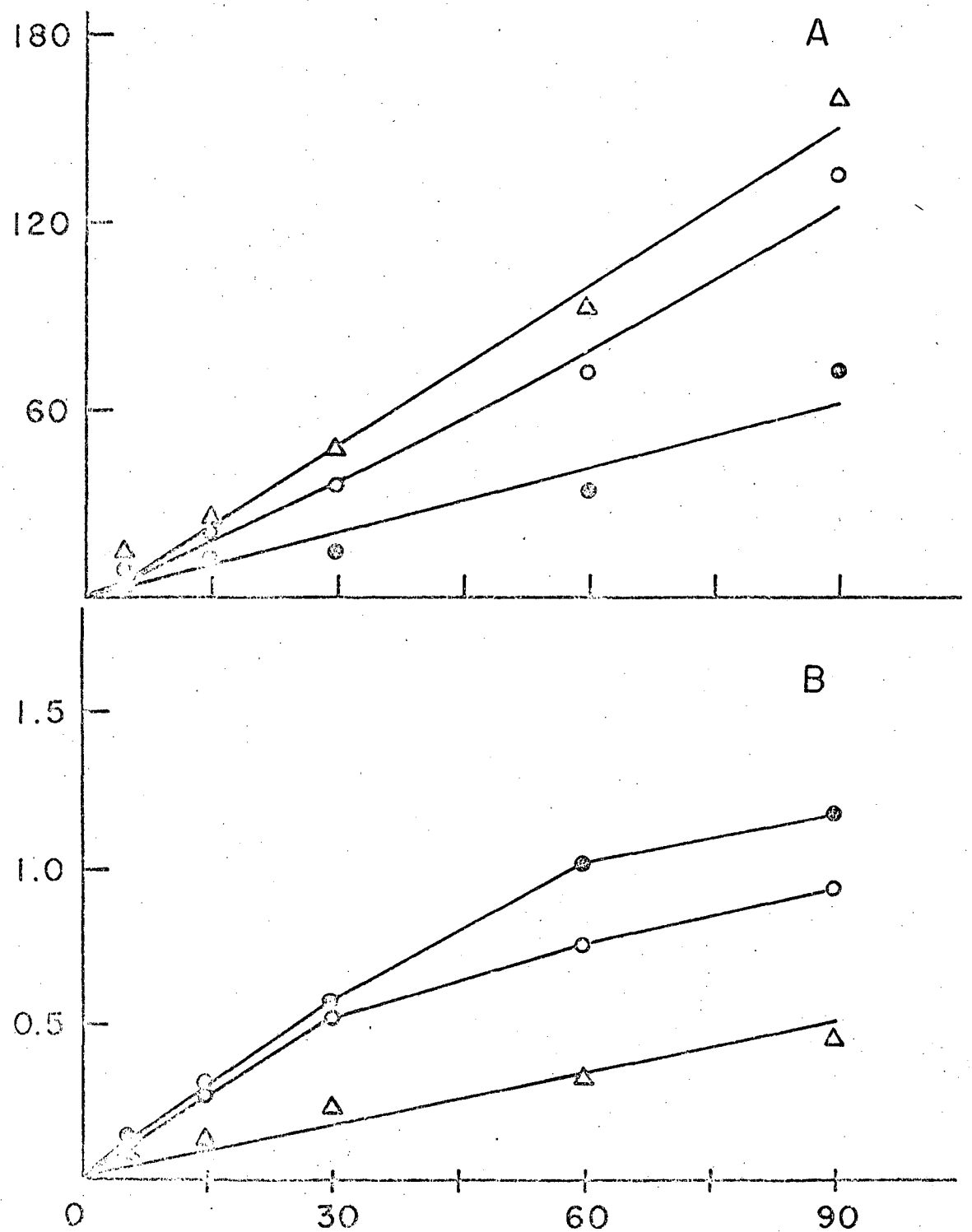

Fig. 1 Neff et al.

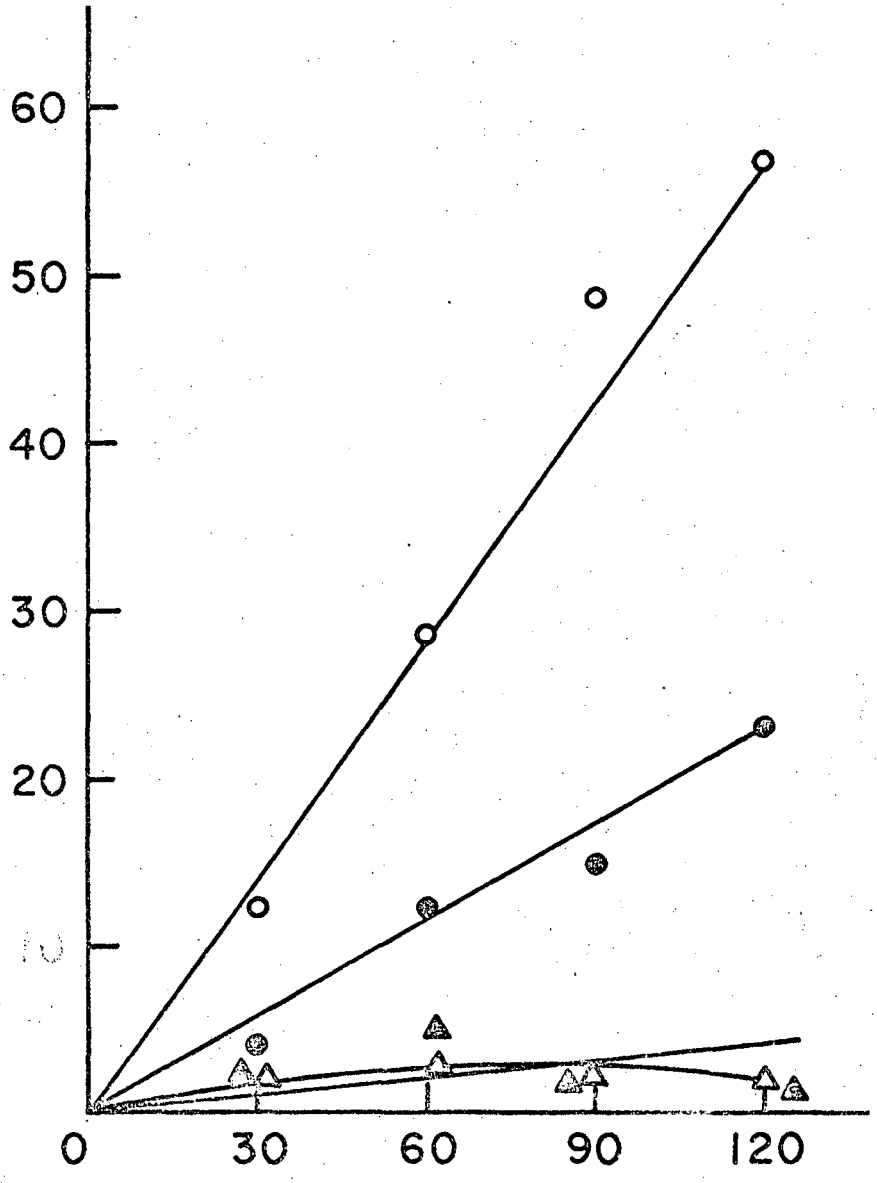

Fig. 2 Neff et al.

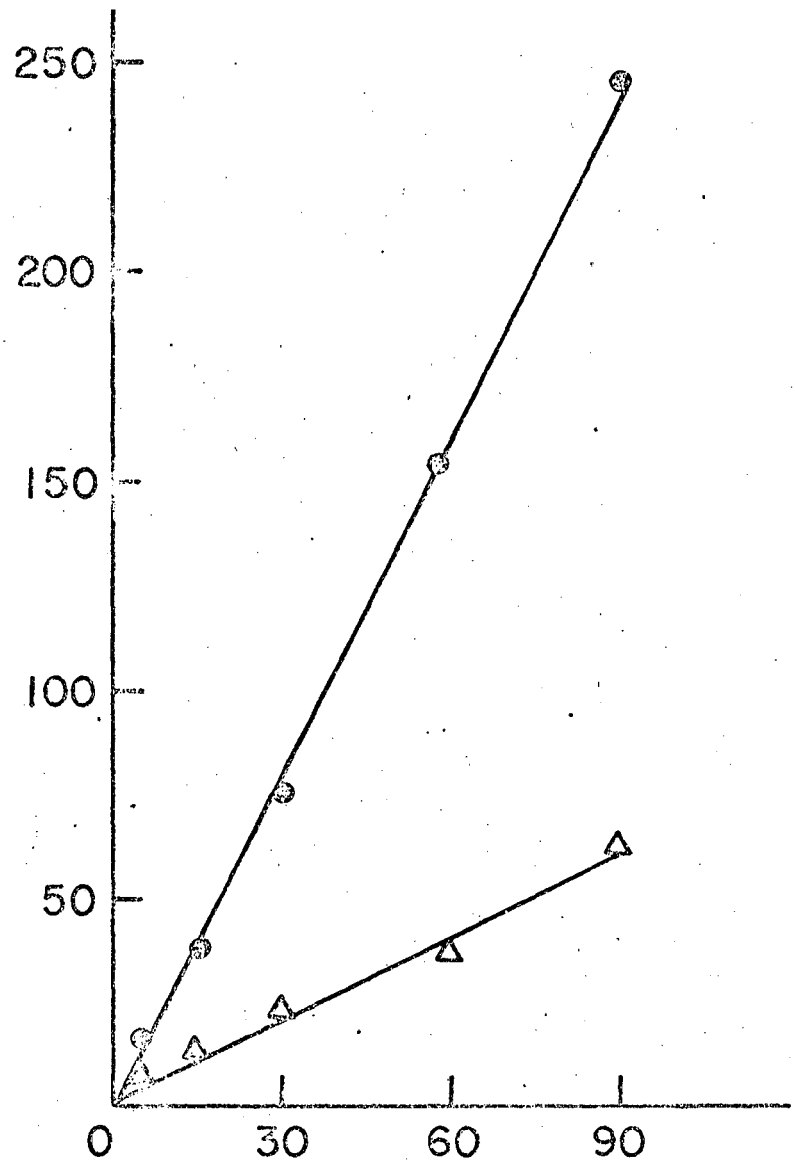

Fig. 3 Neff et al.

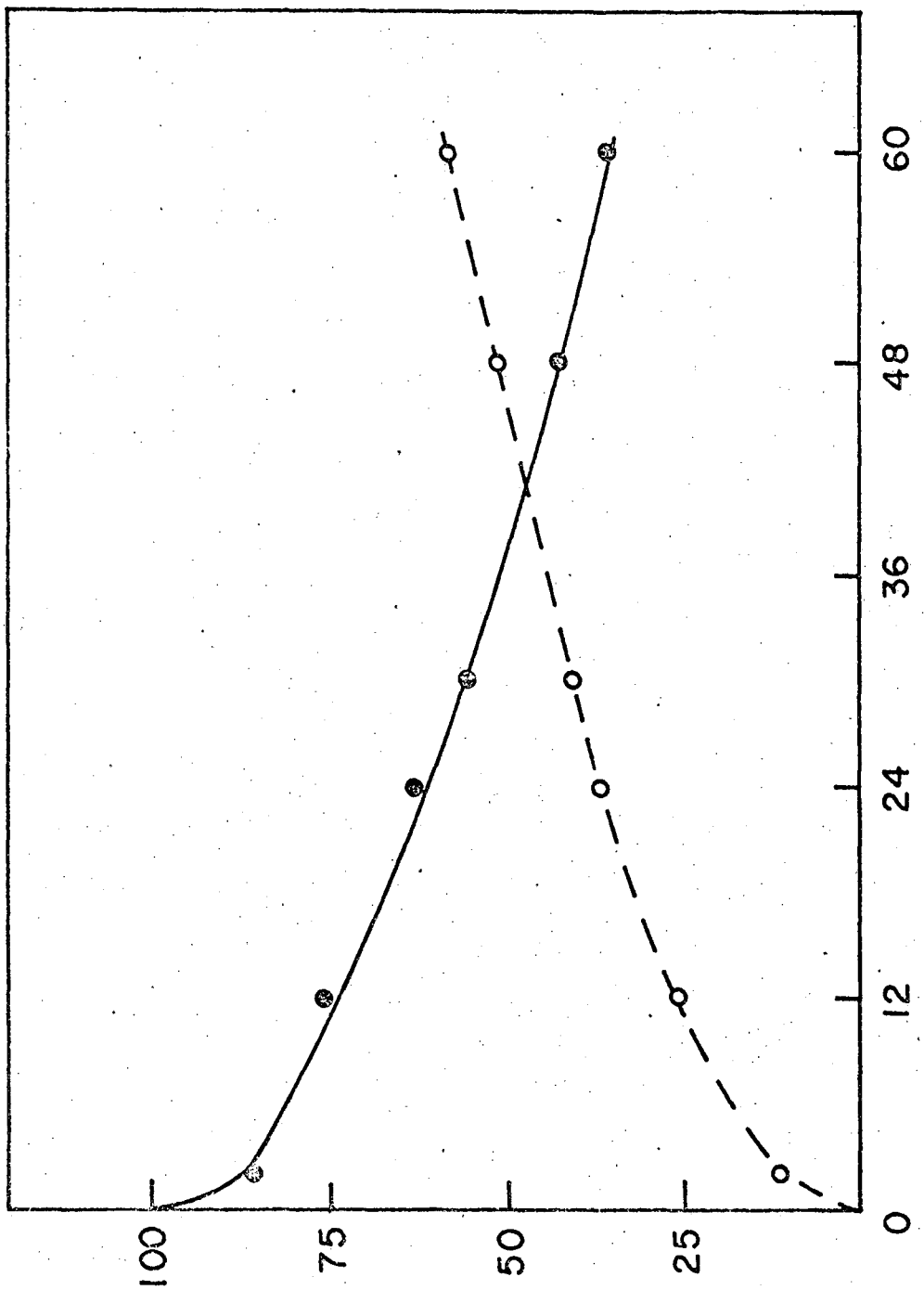

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Fig. 4 Neff et al.

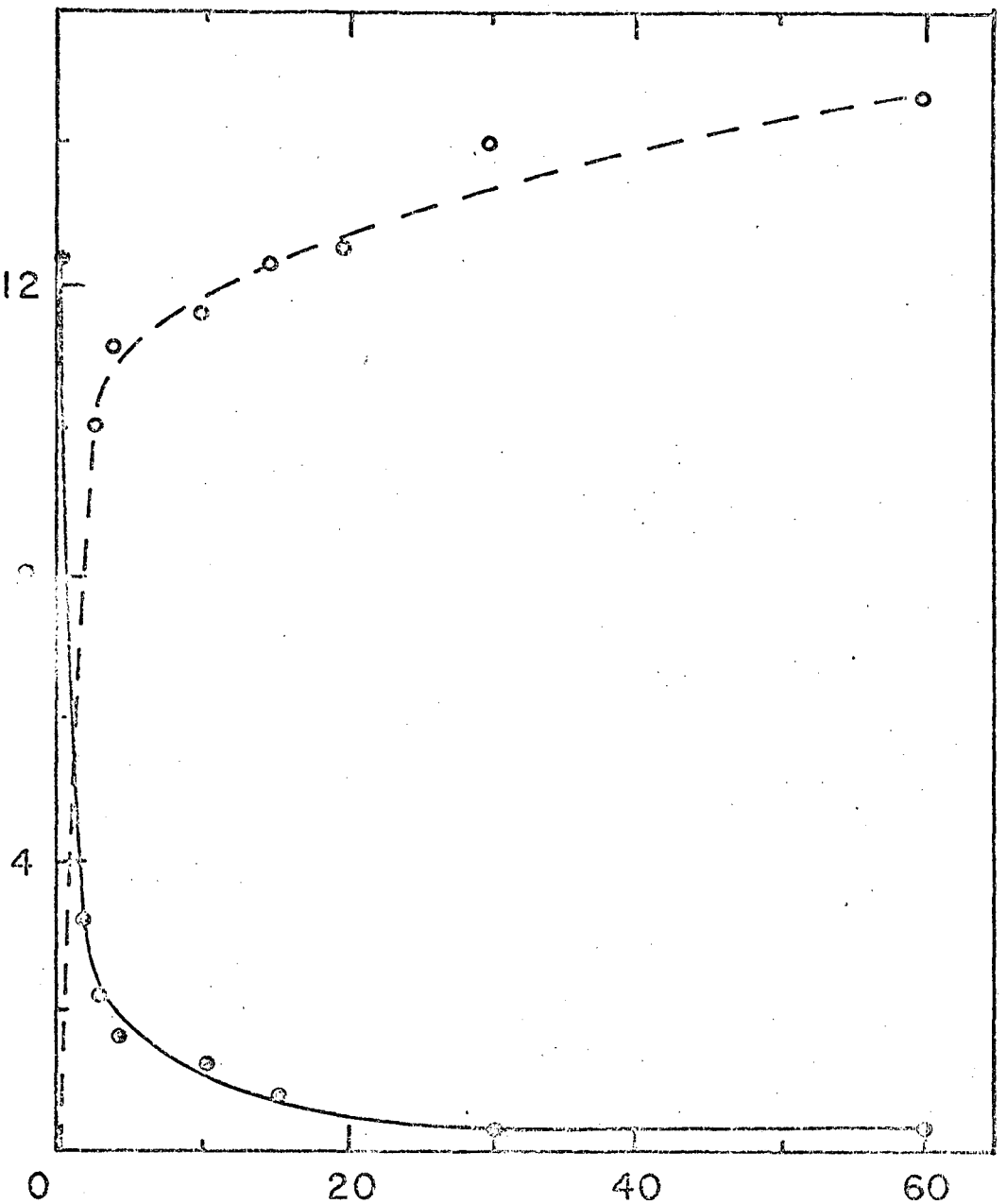

Fig. 5 Neff et al

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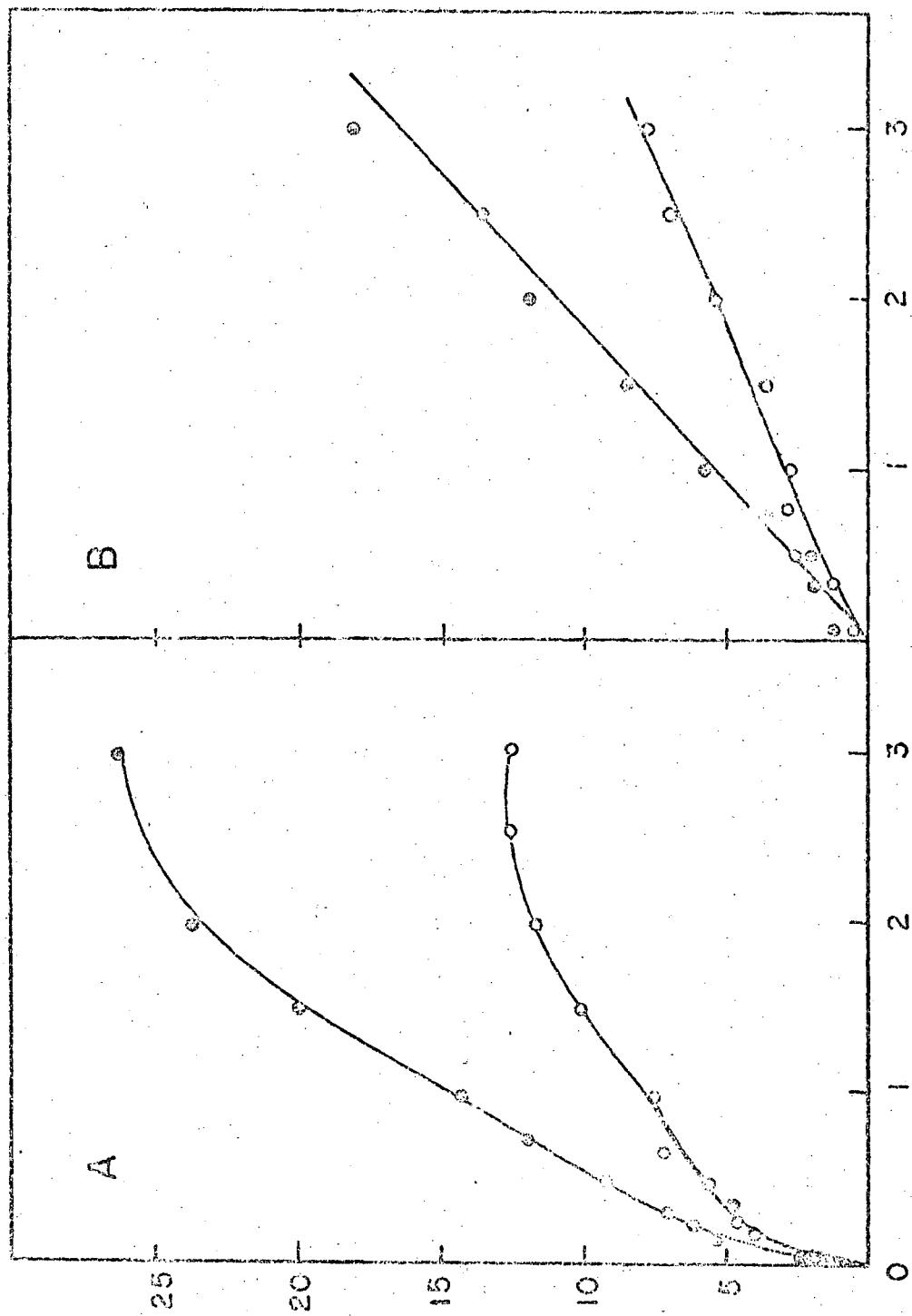

Fig. 6 Neff et al.

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