ARGINASE, AN ANTIMITOTIC AGENT IN TISSUE CULTURE

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INTRODUCTION

Numerous attempts have been made in the past to control the growth of malignant neoplasms by chemotherapeutic agents in addition to the use of accepted methods of surgery and radiotherapy. The experimental approach to the problem in this work differs from most previous ones in that an enzyme, arginase, was used to inhibit the fast growth of fibroblasts and of malignant tissues. If it can be conceived that, amongst other factors, an excess of growth-promoting biological components is responsible for rapid cellular multiplication, then a destruction of such agents might be expected to lead to a retardation of tissue growth. Arginine has been shown to act as a stimulating agent for tumour growth (BACH AND LASNITZKI[1]) and there is also a strong indication that arginine is preferentially utilized by animals bearing malignant tumours (BACH AND MAW[2]). There was, therefore, reason to believe that a removal of arginine from the medium and, possibly, from the cellular fluid of tissue cultures through arginase action would retard the metabolic and mitotic activities of fast-growing cells.

In the meantime, several workers, using crude liver extract (WISWELL[3]) or partially purified arginase preparations (VRAT[4], IRONS AND BOYD[5]) as the source of arginase, reported tumour regressions or a reduction of tumour growth after injection of arginase into tumour-bearing animals.

The experiments described below were designed to ascertain an effect of highly purified arginase preparations on the mitosis of fibroblasts and of Jensen sarcoma cells in tissue culture. The principal difficulty consisted in the preparation of enzyme solutions of sufficient purity to exclude any side effect of non-enzymic material. Arginase has in the past withstood many attempts to isolate it from liver tissue in which it is abundant. In 1946 BACH[7] reported the crystallization of the enzyme. However, the method described proved to be difficult in its later stages and attempts of other workers to repeat the procedure did not always meet with success (MOHAMED[6]). Though, for reasons stated, this seems understandable it does in no way justify MOHAMED's claim[6] that the method could not have been successful in the hands of the originator. In fact, the procedure used in the experiments described below was essentially the original method of BACH and yielded highly purified preparations.

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METHODS

The preparation of arginase

100 g acetoned ox-liver powder were extracted with 0.1% manganese sulphate, maintaining the pH between 7 and 8. Non-enzymic material was removed by heating at 51° at pH 6.1 and by further precipitation with 0.4 volumes of ice-cold acetone. Subsequently enzymic protein was precipitated with further 0.6 volumes of acetone. The enzymic precipitate was redissolved in 0.01% $CoCl_2$ solution of one third of the original volume. The acetone was then removed by dialysis against 0.01% $CoCl_2$. This step was followed by a further removal of non-enzymic material through heating at 60° for 20 mins. The greater part of the liver pigments was now separated from the enzymic mixture by the precipitation of the former with zinc sulphate of a final concentration of 0.025%. The mixture, now practically colourless, was dialysed against 0.01% cobalt chloride and repeatedly fractionated with ammonium sulphate. At ammonium sulphate saturations between 50% and 60% the enzyme separated out at room temperature. The "purity" of the enzyme fractions was followed through the various stages of the preparation. "Purity" was defined as "activity" divided by the nitrogen content in mg of one ml solution. "Activity" was defined as microliter urea-CO_2 liberated in 10 minutes at 30° by one ml enzyme solution. The test method described by BACH[7] was used, though the addition of cobalt salt to the experimental mixture was omitted.

When in previous preparations the enzyme solution reached a "purity" value of approx. 23,000, protein crystals appeared on concentration of the solution. All enzyme preparations used in the tissue culture experiments had a "purity" value of more than 18,000. For use in tissue cultures the purified enzyme solution was completely freed from ammonium salts by exhaustive dialysis against saline-containing 0.01% cobalt chloride; the latter was added as an activating and stabilising agent.

Inactivation of arginase preparations. The active enzyme solution was treated with an equal volume of 3 *M* acetate buffer of pH 4.6 and left standing for one hour at room temperature. This was followed by exhaustive dialysis against cobalt-containing saline to remove the acetate. While no precipitation of protein occurred during this treatment, the complete inactivation of the enzyme was verified by activity tests. The protein concentration of the inactivated enzyme solution was adjusted to that of the active solutions. When several concentrations of active solutions were used in the tissue culture experiments, the concentration of the inactivated enzyme corresponded to that of the most active enzyme solution.

Application of the arginase preparations to tissue culture experiments. The stock solutions prepared as described above were diluted with embryo extract for use in tissue culture experiments. The highest final concentration that could be used without appreciable toxic effects on the fibroblast cultures was found to have an "activity" of approx. 120 units; solutions with lower activities were used in parallel experiments. One drop of the embryo extract-enzyme mixture was added to one drop of plasma in which the tissue was embedded.

Tissue culture technique

Fibroblast cultures. The tissues used were taken from the choroid and sclerotic of 10 days old chicken embryos. The cultures were grown by the hanging drop method in equal parts of fowl plasma and 15% embryo extract in Tyrode solution. They were incubated at 39° and subcultured every 48 hours for three passages to obtain uniform growth. Fresh embryo extract was used on each culture day. Groups of six cultures each from the 4th passage were selected and matched for use in the enzyme experiments and in the controls. The enzyme was added to the embryo extract, maintaining an extract concentration of 15%. The control cultures were grown in absence of arginase. The control and experimental cultures were incubated for 24 hours, after which they were fixed in Susa, stained with Heidenhain's haematoxylin and mounted *in toto*.

The total number of mitotic cells was counted both in the enzyme-treated and in the control cultures. The mitosis of the treated cultures was expressed as percentage of that of the controls. The phase distribution was arrived at by counting the individual phases of the mitoses and by expressing them as percentage of the total mitotic count.

Jensen sarcoma cultures. The Kodak-film-recording technique was used. The cultures were started by the hanging drop method, described above. The medium consisted of one part chicken plasma, three parts rat serum and one part 15% embryo extract. For the film analysis selected cultures of the 4th passage were transferred to Carrel flasks and were grown in a coagulum consisting of chicken plasma, Tyrode and mammalian serum in equal proportions. The coagulum was allowed to clot for three hours before adding the embryo extract (15%). The flasks were incubated for 48 hours, the embryo extract was then removed, the culture washed in Tyrode, after which fresh extract was added. Then pictures were taken for several hours as controls. The embryo extract was again removed and was now replaced by embryo extract containing arginase.

The growth and behaviour of the culture was recorded cine-microphotographically for a period of 26 hours. A picture was taken every 6 minutes on a Kodak-recording film with an automatic camera representing a modification of the one described by WILLMER AND JACOBY[8]. The apparatus

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consisted essentially of an inverted microscope with the objective (4 mm, 0.65 n.a.) occupying the usual position of the condensor. The light of an electric bulb fitted into the tube of the microscope was deflected on the film through a No. 10 eyepiece. A heat-absorbing fluid filter was placed between light source and culture. The microscope was fitted into a thermostatically controlled incubator set at 39°. The mitotic index was evaluated by counting the mitoses occurring in each photograph and by a total cell count at the end of every 6th hour. The number of hourly mitoses was calculated from the number of the cells present.

This technique allows the observation and recording of cell divisions and cell migration over a long period at regular intervals, though only low-power microscopy can be applied.

RESULTS

Experiments with fibroblasts

Mitotic inhibition and cytological observations. Table I summarises the experiments with fibroblast cultures treated with arginase. In each case a control experiment without addition of arginase was carried out. The "activity" of the undiluted arginase preparations used in the various experiments was adjusted to approximately 120 units (see Methods). In some cases (exps. VII and XV) parallel experiments were carried out with further diluted preparations. As is to be expected, the degree of inhibition diminished with high dilutions of the enzyme. Undiluted enzyme preparations appeared to reduce the growth area while with diluted preparations the outgrowth was normal or almost normal. The inhibiting effect was accompanied by cell oedema except in the experiments with high enzyme dilutions. Pyknotic nuclei were observed with undiluted enzyme preparations. The only cytological feature which was generally seen in conjunction with mitotic inhibition was the occurrence of clumped metaphases. This was observed irrespective of the degree of enzyme dilution. The greatest number of clumped metaphases was found in exp. XVb.

TABLE I

MITOTIC INHIBITION AND CYTOLOGICAL OBSERVATIONS WITH ARGINASE-TREATED FIBROBLAST CULTURES

Exp.	*Relative dilution of enzyme preparation*	*% Mitotic inhibition*	*Observations*
II.	none	74.8	Reduced outgrowth; slight oedema in resting cells; clumped metaphases.
III.	none	84.1	
V.	none	74.0	Greatly reduced outgrowth; pyknotic nuclei; oedema in resting cells; clumped metaphases.
VII a.	none	74.5	Little outgrowth; flattened, disconnected resting cells with pyknotic nuclei; clumped metaphases.
VII b.	1:10	71.6	Slightly reduced outgrowth; slight oedema in resting cells; some clumped metaphases.
VII c.	1:100	49.0	Normal outgrowth and normal resting cells; some clumped metaphases.
XV b.	1:50	59.1	Normal outgrowth, slight oedema in resting cells; nearly all metaphases clumped.
XV c.	1:200	44.1	Normal outgrowth; slight oedema in resting cells; some clumped metaphases.
V.	none, inactivated	0	Normal outgrowth; no abnormalities.
VII a.	none, inactivated	7.5	Normal outgrowth; no abnormalities.

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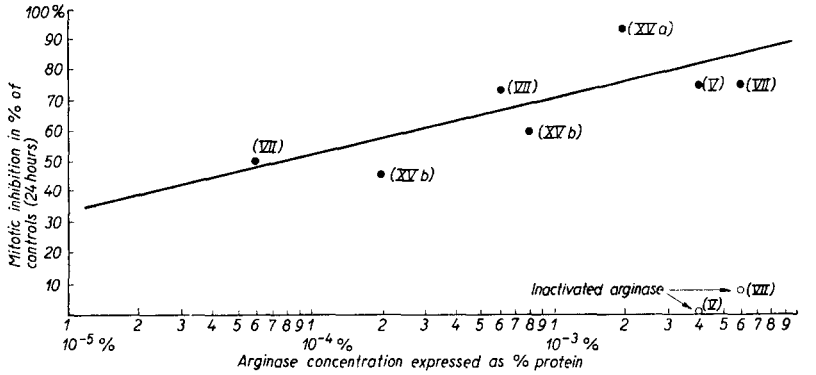

Fig. 1. The effect of the enzyme concentration on the mitotic inhibition of fibroblasts.

Mitotic inhibition and protein concentration. The intimate relationship between protein concentration and inhibitory action of the enzyme is seen in Fig. 1. It is noteworthy that the points referring to different enzyme preparations (as indicated by different Roman numerals) fall close to the line; they were made and applied at different times over a period of two years.

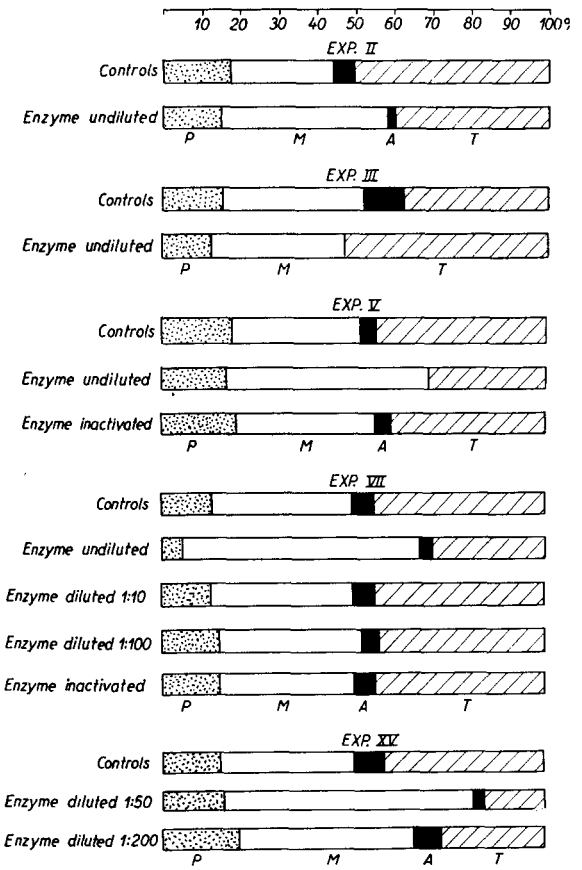

Fig. 2. Phase distribution of arginase-treated fibroblast cultures. Activity of undiluted enzyme: approx. 120 units. P = prophase; M = metaphase; A = anaphase; T = telophase

Effect of inactivated enzyme preparations. Conclusive evidence that the inhibitory effect on mitosis was due to enzyme action and not to a toxic effect of unspecific proteins was secured by experiments with inactivated enzyme solution. In the experiments V and VII the undiluted enzyme preparations were inactivated prior to their administration to the cultures. In these cases no inhibitory effect on mitosis was observed and the cytological picture did not differ from that of the controls.

Effect of arginase on phase distribution. The phase distribution (Fig. 2) shows a tendency to accumulation of cells in metaphase in cultures treated with arginase. This occurs particularly when the culture is treated with arginase preparations of high concentration. On the other hand, the phenomenon was not observed in experiment III where undiluted enzyme was used.

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Experiments with Jensen sarcoma cultures

Preliminary experiments on the effect of arginase on Jensen sarcoma cultures are recorded in Fig. 3. There were carried out with the Kodak-recording technique. In Section A of Fig. 3 the percentage mitosis varied within normal limits during the 26 hours period of the experiment. The variations were similar when inactivated enzyme was added to the culture after five hours, *i.e.* no effect of the inactive solution was detectable. In Section B the variations are similarly normal up to the 10th hour. At this point embryo extract containing active enzyme was added, whereupon the percentage mitosis markedly dropped to reach zero value in the latter part of the experimental period.

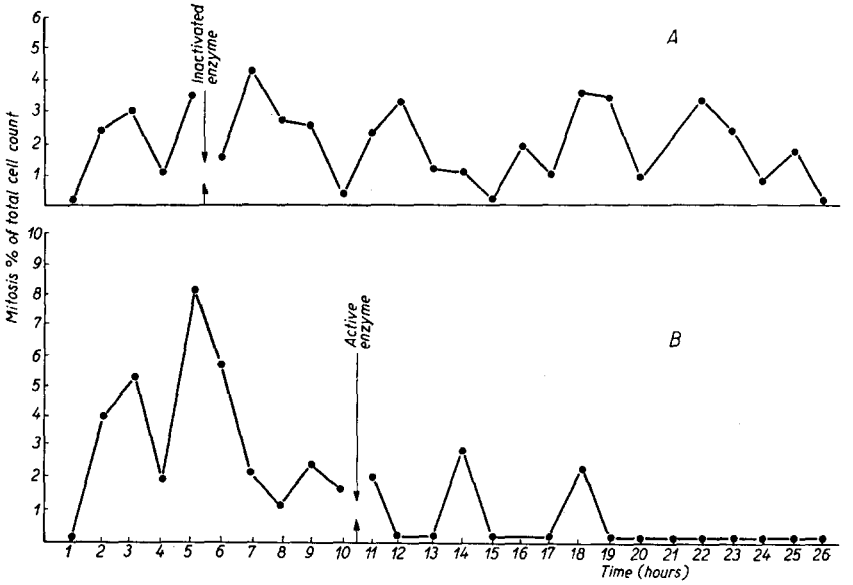

Fig. 3. The effect of arginase on Jensen sarcoma (Expts. XII and XIII). A = inactivated enzyme; B = active enzyme

DISCUSSION

The presence of a highly purified preparation of arginase enzyme in concentrations as low as $6 \cdot 10^{-5}$ % in the medium of fibroblast and of Jensen sarcoma cultures resulted in a strong mitotic inhibition of the cells. Because of the high specificity of arginase with regard to its action on arginine, the observed effect must have been solely due to the destruction of arginine. Only free arginine could have been attacked by the enzyme since it was found elsewhere (BACH[9]) that arginase does not catalyse the decomposition of peptide-linked arginine. The simplest explanation of the inhibitory effect of arginase on cell mitosis may therefore be sought in the destruction of a cell nutrient which may play an essential part in the metabolism of fast-growing embryonic and of malignant cells. In this way the enzyme action may eventually lead to a low arginine level of the culture.

Since it is difficult to picture a permeation of the cell wall by the protein, the enzymic attack must have been directed against free arginine extruded from the cell or present

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in the plasma and in the embryo extract of the medium. That the enzymic effect is not solely concerned with the destruction of the arginine present in the embryo extract, is seen from the fact that an inhibitory action of arginase was also observed when the embryo extract was replaced by Tyrode solution.

An unspecific toxic effect of added protein on the tissue cultures can be excluded as the cause of mitotic inhibition since the addition of the inactive enzymic protein to the culture medium permitted a rate of mitosis identical with that of the control cultures. Observations on the phase distribution and on the cytology of such cultures also showed complete similarity to those made with the control cultures.

It can be argued that the deprivation of an organism from one of its essential metabolites could, besides retarding an excessive growth rate, also lead to a serious interference with the maintenance of its basic metabolism. However, it was observed in these experiments that enzyme concentrations could be found which, while reducing mitosis, did not interfere with the resting cells and only slightly affected the outgrowth of the tissue. Though no detailed cytological studies were made, there is an indication that, at least with higher enzyme concentrations, the cell division may be arrested in the metaphase. Thus the experiments here described represent an attempt to control the mitosis of fast-growing embryonic and of malignant cells *in vitro* by enriching the culture medium with an enzyme which in turn destroys an essential cell nutrient.

For the conditions prevailing *in vivo* it may be of particular interest that the action of arginase appears to be controlled by the adrenal cortex (FRAENKEL-CONRAT, SIMPSON AND EVANS[10,11]) and by ACTH (VRAT[12]). In this way the intact organism may be in a position to adjust, within limits, the enzyme activity. With sufficient arginase at its disposal the organism may therefore be able to regulate the arginine level of the body fluids and possibly to readjust metabolic disturbances caused by the excess of a growth-stimulating nutrient. The increase of the inhibiting effect of arginase on tumour growth through simultaneous injections of arginase and ACTH into tumour-bearing mice, as reported by VRAT[12], confirms this view.

SUMMARY

1. The purification of arginase from ox liver is described.

2. Highly purified arginase, added to the medium of fibroblast and of Jensen sarcoma cultures, caused a strong mitotic inhibition. The inhibition in the fibroblast cultures was almost complete at enzyme concentrations of 10^{-3}% and was still approximately 50% at concentrations between 10^{-4} and 10^{-5}%. An enzyme preparation with an activity of 384 units completely inhibited Jensen sarcoma cultures.

3. The degree of inhibition was related to the activity of the enzyme. When inactivated enzyme was added to the cultures in concentrations as high as the most active preparations, the rate of mitosis was identical with that of the control cultures grown in absence of arginase. The inhibitory effect of arginase was therefore due to a specific enzymic action.

4. Cytological observations revealed reduced outgrowth and an effect on resting cells when higher enzyme concentrations were used. With lower enzyme concentrations which still markedly inhibited mitosis, the outgrowth was normal and the resting cells were unaffected. No abnormalities were observed in experiments with inactivated enzyme preparations. With active enzyme preparations the phase distribution indicated an arrestment of cells in metaphase in most cases.

5. The significance of the control of arginase by the anterior pituitary and the adrenal cortex, as reported by other workers, is discussed.

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RÉSUMÉ

1. La purification de l'arginase du foie de boeuf est décrite.

2. De l'arginase très purifiée, ajoutée au milieu de culture de fibroblastes et de sarcome de Jensen, inhibe fortement les mitoses. L'inhibition, dans les cultures de fibroblastes, est presque totale pour une concentration en enzyme de 10^{-3}% et est encore environ de 50% à des concentrations comprises entre 10^{-4} et 10^{-5}%. Une préparation enzymatique, possédant une activité de 384 unités, inhibe complètement des cultures de sarcome de Jensen.

3. Le degré d'inhibition est lié à l'activité de l'enzyme. Quand on ajoute aux cultures de l'enzyme inactivé, à des concentrations aussi élevées que celles des préparations les plus actives, le nombre des mitoses est le même que dans des cultures témoins réalisées en l'absence d'arginase. L'inhibition par l'arginase est donc due à une action enzymatique spécifique.

4. Des observations cytologiques montrent que des concentrations plus élevées en enzyme réduisent la croissance et ont un effet sur les cellules au repos. Des concentrations faibles, qui inhibent encore nettement les mitoses, n'ont pas d'action sur la croissance et les cellules au repos. L'enzyme inactivé ne produit aucune anomalie. L'enzyme actif bloque dans la plupart des cas les cellules en métaphase.

5. L'importance du contrôle de l'arginase par l'antéhypophyse et le cortex surrénal, qui a été indiqué par d'autres auteurs, est discutée.

ZUSAMMENFASSUNG

1. Die Reinigung von Arginase aus Ochsenleber wird beschrieben.

2. Fügt man Arginase von hohem Reinheitsgrad zu dem Medium von Fibroblasten- und Jensen-Sarkom-Kulturen, so tritt eine starke Mitosenhemmung ein. Bei einer Enzymkonzentration von 10^{-3}% war die Hemmung beinahe vollständig. Bei einer Enzymkonzentration zwischen 10^{-4} und 10^{-5}% betrug sie noch ungefähr 50%. Ein Enzympräparat mit einer Aktivität von 384 Einheiten hemmte Jensen-Sarkom-Kulturen vollständig.

3. Der Grad der Hemmung stand in direkten Verhältnis zur Aktivität des Enzyms. Fügt man inaktiviertes Enzym zu den Kulturen in derselben Konzentration wie diejenige in den Präparaten mit höchster Aktivität, so ist der Ablauf der Zellteilung identisch mit der Arginase-freien Kontrollkulturen. Die hemmende Wirkung der Arginase ist daher einer spezifischen Enzymwirkung zuzuschreiben.

4. Cytologische Beobachtungen zeigen bei höheren Enzymkonzentrationen eine reduzierte Wachstumszone, sowie eine Wirkung auf die im Ruhestadium befindlichen Zellen. Bei geringeren Enzymkonzentrationen, die die Mitosen noch deutlich hemmen, war das Wachstum normal und die Zellen im Ruhestand waren nicht angegriffen. In den Versuchen mit inaktivierten Enzympräparaten wurden keine Abnormalitäten beobachtet. Bei aktiven Enzympräparaten zeigt die Phasenverteilung in den meisten Fällen eine Hemmung der Zellteilung in der Metaphase.

5. Die Bedeutung der von anderen Forschern berichteten Steuerung der Arginase durch den Hypophysenvorderlappen und die Nebennierenrinde wird besprochen.

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