CLXXV. PROTEOLYSIS IN TUMOURS.

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INTRODUCTION.

AMONG the most difficult questions regarding the metabolism of tumour cells are those of the content of proteolytic enzymes in the cell itself and the linkage between the proteolytic functions and other metabolic processes as well as their relations with malignancy.

It has been admitted for a long time [Neuberg and Gottschalk, 1925] that tumour tissue has a high content of so-called autolytic enzymes and also a certain degree of proteolytic activity against proteins of normal tissues (heterolytic function); and this ought to be an explanation for the liquefying and destroying action, which tumour growth exerts on surrounding tissues. There are also some earlier observations of Abderhalden and co-workers [1909; 1910] on atypical peptolytic activities of tumour tissues against some artificial polypeptides.

During the last few years we find the work of Waldschmidt-Leitz and Schäffner [1930], who, applying to the study of tumour enzymes the methods and conceptions of the Willstätter school, found that the cathepsin was abundant in a rat sarcoma and showed during tumour growth an increasing degree of self-activation, *i.e.* it required less and less activation by H_2S for action on gelatin. As activation by H_2S and sulphydryl bodies (SH-glutathione, cysteine, *etc.*) characterises the catheptic enzymes of cells according to the ideas of these authors, their finding would signify that the tumour as it grows contains more and more activators, represented by reducing substances like the foregoing. A relation of these substances and therefore of the increased proteolysis with the lowered oxidation in tumours is consequently supposed.

Krebs on the contrary in an extensive study of proteolytic enzymes of normal and tumour cells could find no special features in enzymes from tumour tissues of man and animals. He tried to demonstrate [1930] that the promoting action of substances containing the —SH group and of HCN on cathepsins was not a true activation but rather the effect of a combination with traces of inhibiting, enzyme-poisoning heavy metals (“*Komplexbildung*”). He then [1931] stated that tumours are to be ranged as to proteolytic activity below some normal organs (kidney in rat, thyroid gland in man), if the cellular enzymes are studied by appropriate methods, *e.g.* if care is taken to enhance

to a maximum the enzymic function by adding to the mixture of tissue extract *plus* substrate (gelatin) a proper concentration of cysteine, which is able to combine with heavy metals, preventing in this way any disturbance of enzymic function due to traces of such metals.

Stern [1931] studied the action of cell enzymes on the proteins of the extract themselves, *i.e.* he developed a method corresponding to the experimental arrangement of autolysis. He measured the undigested protein of the extracts by a nephelometric method, after precipitation with sulphosalicylic acid and HCl. He apparently disregarded the activation by H_2S or by glutathione; and as regards the influence of heavy metals on proteolysis, he found for some of them an inhibiting action, for others a promoting one, which is not in agreement with the ideas of Krebs. A rat tumour (Cori-tumour) showed nearly the same behaviour as the liver of the same species. A clear review of the subject is due to Kleinmann [1931], who emphatically denies any special feature of proteolytic enzymes in tumours.

However, the idea that proteolytic processes should be very pronounced in tumours cannot be lost sight of, as we find also in recent times observations pointing to strong digestive action of tumour cells (Rous sarcoma) *in vitro* on fibrin and also on normal tissues; and every histologist knows the large necrotic, *i.e.* autolytic, processes in tumours, chiefly in those of the small rodents. I may point to the recent work of Price [1931], who was not able to confirm the ideas of Robin [1919], that in malignancy normal surrounding tissue is digested and that the growth builds up its protein from the digestion products; but he found a larger content of soluble N (non-protein-N) in the growth (Brown-Pearce tumour of rabbit) than in the adjacent normal tissue, into which autolytic products diffuse *post mortem*. He concludes: "evidence has been obtained that a malignant growth does not liberate proteolytic enzymes into the surrounding tissues"; but he does not deny the extensive proteolysis in the tumour itself.

EXPERIMENTAL.

Proteinase. General features of action.

The present work is to be considered as an attempt to study the proteolytic function of a tumour (Ehrlich's strain of mouse cancer) in a more natural way than that used by previous investigators, *i.e.* in a way more related to the technique of Stern for normal cathepsins. Like this author, I let the tumour enzymes react on the proteins of the tumour itself (autolytic function): the extracts, prepared according to Waldschmidt-Leitz and Deutsch [1927] with acidified glycerol (one part by weight of fresh tissue, minced and mixed with two volumes of 87 % glycerol acidified with 0.15 % of *N* acetic acid, kept for some hours at 37°, filtered through paper, and kept in ice-box), were diluted with water, brought to a proper p_H and placed in an Ostwald thermostat at 37° for the required time. The enzymes, which are inactive at a high concentration of glycerol and at low temperature, are able to act on the

proteins (about 0.5 % in tumour extracts prepared as above), when the glycerol reaches a high dilution (5 %) and when the system is brought to the proper temperature and reaction. We may then make an estimation of the rate of digestion by many methods. Stern used the nephelometric estimation of the undigested proteins, which seems to me not very reliable, as the dispersion of the precipitate obtained with HCl and sulphosalicylic acid is very variable and therefore the degree of turbidity may not run parallel with the content of undigested protein. I suppose that some nephelometric observations express more the influence which some factors (p_H , foreign substances, *etc.*) exert on the dispersion of proteins precipitated by sulphosalicylic acid than the different degree of protein digestion; at least these two different ways of modifying the turbidity may show themselves in the final result. Besides this a nephelometric method must be excluded when we wish to try the influence of a turbid solution (such as lipoids generally give) on digestion. On the other hand all methods consisting in the estimation of digestion products, and particularly the Van Slyke gasometric method, require rather large quantities of substrate to give sensitive and reliable results; and the digestion must generally last many hours (24) in working with organ enzymes, so giving rise to some disturbing conditions.

After many attempts I found it to be a sufficiently exact method to weigh the protein precipitate obtained with trichloroacetic acid (final concentration, 5 %). This precipitate (undigested protein) is collected on previously weighed filters, thoroughly washed with dilute trichloroacetic acid, then with alcohol (hot and cold) and with ether, dried at 105–110° and weighed.

An example of one of the first experiments is given here.

Exp. 1. Three similar mixtures were made each containing

2 cc. of *M* buffer solution (sodium acetate-acetic acid) at p_H 3.8 + 36 cc. redistilled water + 2 cc. of a tumour glycerol extract (as above).

To mixture (1) 40 cc. of a 10 % trichloroacetic acid solution were at once added.

Mixture (2) remained 2 hours at 37° (water-bath); and then 40 cc. of 10 % trichloroacetic acid solution were added.

Mixture (3) remained 6 hours at 37° and then the same addition was made.

The three precipitates (undigested protein) were collected and washed, then dried and weighed. Results in mg. protein:

From mixture (1) 11.2 (starting-point : original protein content).

From mixture (2) 9.8 (extent of digestion = 12.4 %).

From mixture (3) 5.0 (extent of digestion = 55.5 %).

The final experimental method was generally arranged with 60 cc. total volume of the digestion mixtures, containing 3 cc. of the glycerol extract, 4 cc. of the buffer along with redistilled water and the substance, the action of which was to be tested on digestion. At the moment of stopping the digestion 20 cc. of a 20 % solution of trichloroacetic acid were added. The digestions

were always carried on at 37° (water-bath). When I wished to make a control by Van Slyke's gasometric method 10 cc. were removed from the mixture and treated in the usual apparatus, the remaining 50 cc. being used for the gravimetric method.

The different tumour extracts (old well-developed tumours or young tumours; more or less of necrotic areas; differently treated or untreated tumours) were not always quite alike as regards content of enzymes and of proteins; they showed also some differences in p_H -optimum, which in some cases changed with the age or time elapsed from the preparation. We know from the work of Willstätter and his school that the p_H -optimum of an enzyme depends on many factors.

A tumour extract, made in March 1932 from well-developed (aged 21 days) tumours of 20 mice, gave the following results after 10 days and after 60 days from the preparation (1 tissue + 2 acid glycerol).

Exp. 2. Digestion mixtures of 60 cc. total volume as described above containing 3 cc. of extract. Extent of digestion after 6 hours (%) at 37°.

	Extract 10 days old	Extract 60 days old
p_H 4	0	29
p_H 4.7	29	20.2
p_H 5.17	37	17.5

This extract acted well in the beginning at p_H 4.7–5.1, although the latter value was to be considered not very reliable because some precipitation spontaneously occurred in the mixture after 6 hours' incubation; I therefore took as optimum p_H 4.7, which was used in most of the experiments. Krebs worked at the same p_H in his important investigations. The more acid reaction (p_H 3.7–4) has often given very good digestion, as in *Exp. 1*; but sometimes in fresh extracts inexplicable inhibitions occurred (generally not so strong as in the case given above).

The comparison between fresh and older extracts was all the more difficult in that the amount of protein itself changed somewhat (slow spontaneous breakdown in spite of the high glycerol concentration). The extent of digestion had always to be referred to a new control value of undigested protein.

Table I.

	Extent of digestion at 37° (%).			
Extracts tested:	2 hrs.	4 hrs.	6 hrs.	12 hrs.
A at p_H 3.8	12.4	—	55.3	—
A at p_H 4.7	—	—	34.6	53.5
A at p_H 4.7 (1 month later)	—	—	29.5	—
A at p_H 4.7 (2 months later)	—	—	13.43	—
L.S.C. at p_H 4.7	—	—	29	—
L.M.C. at p_H 4.7	—	—	55.6	—
L.M.C. (19 days later)	—	—	11.7	—
R.	11.5	20.4	35.5	45
R. 2	9.5	18	32	40.2
R. 3	13	25	52	59.5

As regards the course of digestion, I may state that there are differences between the various extracts: if we take the average course at p_H 4.7 (the one usually adopted), we find 10–15 % digestion in the first 2 hours, 30–35 % digestion after 6 hours, and a final value of 50–60 %, even after 24 hours at 37°.

The behaviour of some different extracts is summarised in Table I.

These results are similar to those obtained by Stern [1931, Fig. 5] on a Cori-tumour of the rat.

Activation with H_2S and cysteine.

After passing a current of H_2S through the digestion mixture at room temperature for half an hour, no clear activating influence of H_2S could be detected by precipitation of the undigested protein. The estimation of precipitated protein by weight is rendered difficult in the mixture treated with H_2S by the precipitation of some sulphur, which may be largely removed by washing on the filter with carbon disulphide.

The result with 0.005 *M* cysteine hydrochloride was more definite. An extract gave an 82 % digestion in the presence of cysteine after 2 hours at 39° against 51.7 % in the control mixture without the amino-acid. Another experiment gave the following result.

Exp. 3. Three mixtures were prepared each containing 4 cc. buffer solution at p_H 4.7 and 3 cc. tumour extract (aged 23 days), made 1 : 4 with acid glycerol; to No. 3 were added 3 cc. of 0.1 *M* cysteine hydrochloride (Roche).

Mixture (1) was at once treated with 20 cc. of 20 % trichloroacetic acid; mixtures (2) and (3) remained 7 hours at 37° and were then treated with trichloroacetic acid.

Results: mg. of undigested protein:

From (1) 5.2.

From (2) 4.6 (11.5 % digestion).

From (3) 2.6 (50 % digestion).

Other experiments with extracts more active in themselves gave less striking, but still clear results. Cysteine is able to increase the activity of cathepsin in tumours as well as in normal tissues; and this influence may be observed also using as substrate the protein of the extracts themselves, contrary to the statement of Kleinmann.

Effects of lecithin.

Here also difficulties occurred in the estimation of undigested protein, because the phosphatide remained strongly adherent to the precipitate and a very long, tiresome washing with alcohol and ether was necessary.

Exp. 4. Six mixtures were prepared (total volume 60 cc.) each containing 4 cc. buffer solution at p_H 4.7; 3 cc. tumour extract A (made 1 : 3 with acid glycerol). To Nos. 1, 2 and 3, 3 cc. of a 1 : 3 dilution of ethyl alcohol in redistilled water were added and to Nos. 4, 5 and 6, 3 cc. of a 1 : 3 dilution in

redistilled water of a 2 % alcoholic solution of lecithin (Merck); *i.e.* 0.02 g. of lecithin were present in the mixture.

Mixtures (1) and (4) were at once treated with 20 cc. of a 20 % solution of trichloroacetic acid.

Mixtures (2) and (5) remained 6 hours and mixtures (3) and (6) 19 hours at 37°, and were then treated with trichloroacetic acid.

Results: mg. of undigested protein:

From (1)	15.
From (2)	9.8 (34.6 % digestion).
From (3)	7 (53.5 % digestion).
From (4)	14.2.
From (5)	7.8 (45 % digestion).
From (6)	6.4 (54.9 % digestion).

From this experiment a slight accelerating effect of the lecithin may be deduced. In other cases the effect was still less evident but never quite negative or inhibiting. It may be remembered that Pianese [1917] assumed an activating influence of lecithin on intracellular enzymes in cancer to explain his histological observations.

Dipeptidases and aminopolypeptidases.

The action of tumour extracts on dipeptides and polypeptides was investigated according to the usual methods of Waldschmidt-Leitz and co-workers.

The dipeptidases were tested on leucylglycine (from the laboratory of Prof. Waldschmidt-Leitz, Prague); and the aminopolypeptidases on leucylglycylglycine (Roche). The solutions of the peptides were prepared as follows.

3.764 g. of leucylglycine or 4.9 g. of leucylglycylglycine were dissolved in 40 cc. of a 1 : 1 mixture of *N* solutions of ammonium chloride and ammonia; 20 cc. of distilled water were added, the dissolution being helped by warming. 5 cc. of *N* acetic acid solution were added and the volume made up to 100 cc. with water. These solutions (p_H 8) contain 0.001 *M* peptide.

For the experiment 5 cc. of peptide solution were mixed with 4 cc. of redistilled water and 2 cc. of extract; 5 cc. were at once taken and used for a blank estimation, and a second estimation was made on 5 cc. after 1 hour at 37°. The estimations were made according to the alkalimetric method of Willstätter and Waldschmidt-Leitz [1921] in alcohol (5 cc. of digestion mixture were added to 100 cc. of neutralised absolute alcohol and titrated with alcoholic 0.1 *N* KOH with phenolphthalein as indicator). The extent of digestion is given by the increase of carboxyl groups set free and therefore by the difference between the estimations before and after digestion (cc. of 0.1 *N* KOH).

Three extracts from cancer of mice were investigated as to content of dipeptidases. In each group of mice the livers were also taken and extracts with acid glycerol prepared and tested in the same way, so that a comparison of peptidase content in tumour and in liver is possible.

Results. Dipeptidase contents (cc. of alcoholic 0.1 N KOH).

Extracts (set of mice)	Tumour	Liver
L.S.C.	1.60	2.90
L.S.	2.15	2.65
L.M.	0.70	0.65
Average	1.48	2.06

As regards aminopolypeptidase I tested only an extract from the tumours of 5 mice submitted to a treatment with lecithin (Merck) and found none at all. The livers of the same animals showed a content corresponding to 0.6 cc. of 0.1 N KOH.

My results may be well compared, on the ground of the identity of methods of estimation, with the results of Waldschmidt-Leitz and Schäffner [1930] on a rat sarcoma: they found values of dipeptidases which varied from 1.12 to 1.50 cc. 0.1 N KOH and averaged about 1.30. My results were very similar, but slightly higher; the epithelial nature of the tumour investigated by me may account for the small difference. I cannot state whether there is an increase with progression of tumour growth, because I examined in every case well-developed cancers. There may be some importance in the complete absence of aminopolypeptidases in contrast to the high content found by the investigators just mentioned, and also to the content of the liver of the same animals. This point deserves further research.

*Attempts to modify the enzymes of tumours by treatment
of the tumour-bearing organism.*

Some of the foregoing data were drawn from two experiments bearing on the effects of lecithin treatment of tumours. In a long series of papers from 1926 onwards I had tried to study the influence of many lipid fractions on the growth of tumours, and many experiments pointed to an inhibiting action of lecithin, parenterally introduced during the development of mouse cancer [1930; 1931]. As I had the opportunity a few months ago of using a distearyllecithin prepared by Grün and Limpächer [1923] in my investigations [1932] on the effects of phosphatides on proteolytic enzymes, and was able to obtain more of this interesting compound through the kindness of Dr Limpächer, I submitted some cancerous mice to treatment with it. The small quantity of synthetic lecithin at my disposal was insufficient for the treatment of a large series of mice.

Of 11 cancer-bearing mice (graft on May 10th) 5 were kept as control; 6 received every two days from May 17th till 27th an injection of 1 cc. of a 1 % solution in water of the synthetic lecithin, which after thoroughly grinding and by gentle warming gives a stable solution in distilled water. Every mouse received 0.06 g. in the course of the 10 days' treatment. By weighing the tumours of the untreated and treated mice (which were all killed on the 27th) and dividing the weight of every tumour by the number of days elapsed since the grafting, we get the growth index or daily increase of tumour mass.

The average growth index was 0.084 g. for the treated mice; 0.138 g. for the untreated. The synthetic phosphatide gave therefore an inhibition of the tumour growth of about 39 %.

Extracts were prepared from the tumours of both groups of mice, taking great care to work quantitatively and to obtain two equally diluted preparations.

They were tested as to catheptic (proteinase) function in the usual way and as to dipeptidase content; the livers were also worked up and tested. Some of the data given before were drawn from this experiment; they may be summarised here as follows.

Tumour extracts from untreated mice gave a protein autolysis of 29 % in 7 hours, and a dipeptidase content of 1.60 cc. 0.1 *N* KOH.

Tumour extracts from treated animals showed less proteolytic activity (20 %) and more dipeptidase content (2.15 cc.).

Liver extracts gave quite opposite results: the catheptic function on their own proteins seemed to be a little higher (with some uncertainty) in treated mice; the dipeptidase function was here a little reduced. At any rate the differences were smaller than in the tumours.

Another experiment was undertaken with lecithin *ex ovo* (Merck), which had to be dissolved in alcohol (2 %), the solvent evaporated on the water-bath and the fatty material taken up with water for injection. The inhibiting action on cancer of a treatment with a total of 0.05 g. for each mouse was less pronounced here than in my previous researches [1930], when I injected lecithin together with pig-serum: 23.7 % inhibition was observed in 6 treated mice against 4 untreated. The estimation of the proteolytic function gave a lessened activity in tumours from treated mice, just as in the experiment with synthetic lecithin (30 % digestion in 7 hours against 50 %). Dipeptidases were not compared for lack of material.

It may be said that in two experimental series dealing on the whole with 20 mice and with 4 extracts from their tumours, a treatment with lipoids, which was able to restrict the growth of a very virulent tumour, also reduced the content of proteinases or rather modified the conditions of the system proteinase *plus* protein of tumour tissue in a way which led to a lowered digestive activity. The enzymes of a normal organ (liver) seemed to be less and quite independently affected.

DISCUSSION.

These experiments are favourable to the opinion that the proteinase and "ereptase" activities in tumours do not differ essentially from those already well known in normal tissues. At least I was not able to discover a single specific feature. Quantitatively considered, they are to be ranged rather high, although not higher, and sometimes indeed lower than the corresponding functions of some normal organs. I would point out that it is not right to compare an enzymic function of a tumour with the same function in any normal tissue other than that which itself gave origin to the tumour.

That proteolysis has an importance in tumours during life is well shown by the high digestive power of tumour cells *in vitro* (Rous sarcoma) and by the very common observation of large autolytic and necrotic areas. However, the relation to cell multiplication and to growth is not clear. I may state that in two sets of experiments a decrease of growth velocity seemed to be accompanied by a decrease of proteinase content and once by an increase of dipeptidase; but it would be imprudent to lay too much stress on such differences between extracts from parallel groups of animals. I may rather state that the results of *in vitro* experiments are often the reverse of those of *in vivo* experiments. Thus while lecithin increases the proteolytic activity of extracts from normal organs on gelatin, as I found before [1932], and also of tumour extracts on their own proteins, as is shown in this paper, lecithin itself, the synthetic non-physiological compound as well as the preparation *ex ovo*, diminishes the growth of tumours and the autoproteolytic activity of the tumour tissue. The lecithin therefore influences the tumour not only or not chiefly directly, but also indirectly, through the metabolism of the tumour-bearing organism. The content of proteinases and dipeptidases in the tumour may change under a lecithin treatment of the animals independently of the content in a normal organ (liver). The proteinases as studied in an autolytic experiment need no special activator: they are active by themselves in tumours as well as in normal tissues and produce in 6 hours an average digestion of 35 % of the tissue proteins. In other words the extracts prepared with acid glycerol [Waldschmidt-Leitz and Deutsch, 1927] contain a complete enzymic system. However, some substances like phosphatides and cysteine can enhance the digestive activity more or less. These substances are not necessary activators, but rather accelerators of the enzymic function. It may be that compounds containing the —SH group are already present in the extracts and work as true activators; then the added cysteine would complete the supply of it. And it may also be admitted that the different degrees of activity of the extracts depend on the amount of natural activators, *e.g.* glutathione, already present. But I have derived no direct proof from my experiments of such a general constitution of the enzymic system in tumours. Some experiments would suggest that cysteine, which generally facilitates the proteolysis, is able under not well defined conditions to reverse this function and to produce a new reconstitution of protein. As the experimental demonstration of this very important phenomenon is not yet conclusive, I preferred not to report the experiments and to reserve this point for further investigation.

I would conclude that the strong proteolytic functions, often exhibited by living tumour cells, are not the expression of special characteristics of the enzymic equipment of the cell but rather a consequence of the biological derangement, which allows the cell to use its normal enzymes in an abnormal way. As with many other aspects of malignancy we must look at the vital behaviour more than at the purely chemical constitution of cells. As the circulation in tumours is somewhat defective, it may happen that some

derivatives formed by the breaking down of the very labile tumour cells are not removed so readily as in normal tissue; and they may on the one hand check the further disintegration (which we know is possible chiefly from the work of Rona *et al.* [1930], but also on the other hand sometimes facilitate proteolysis, if, among the products of the disintegration of cellular lipoids, proteins and lipid-protein compounds, substances predominate which may enhance the catheptic function, as do lecithins and cysteine.

A very complicated situation arises which cannot be expressed by a general law and explains the differences shown by tumours of different origin and age or submitted to some treatment.

SUMMARY.

1. The cancer of mice (Ehrlich's strain) gives extracts with acid glycerol, which may be easily investigated as to proteolytic activity on their own proteins (autolytic experimental type). The general features of the tumour proteinases have been described. The optimum p_H changes a little according to the age of tumours and of extracts; generally the digestion experiments were carried on at p_H 4.7. The activity of proteinases in tumours is not higher than in some normal organs, although an exact statement on this matter (comparison with the organ from which the tumour growth started) is not easy. The digestion averages generally 35 % of the extract protein in 6 hours at 37°.

2. Lecithin has a slight accelerating action on proteolysis.

3. Cysteine has a more or less pronounced accelerating action.

4. A variable content of dipeptidases was found; aminopolypeptidases were lacking in the one series examined, while the livers of the same mice contained them.

5. Treatment of the tumour-bearing mice with synthetic lecithin and with lecithin *ex ovo* delayed the tumour growth, and produced some modifications in the enzyme content, which are briefly discussed. These modifications do not run parallel with modifications of enzymes in normal organs (liver).

6. The proteolytic enzymes (cathepsins) in the investigated tumour show no special features in comparison with the enzymes of normal tissues, although their function in the living tumour cells may be in some way peculiar. The malignant cell may utilise a normal enzymic equipment in an abnormal way.

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