CLXIII. AUTOLYSIS IN MALIGNANT AND NORMAL RABBIT TISSUES¹.

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IN connection with proteolytic change in malignant growths Robin [1919] arrived at the following conclusions, based upon an analysis of malignant and normal human tissue.

(1) There is "a decrease in the insoluble nitrogen and an increase in the soluble nitrogen in the 'relatively healthy' regions bordering upon the growth as compared not only with the most cancerous regions but also with normal healthy tissue."

(2) There is "an increase in the insoluble nitrogen, *i.e.* protein nitrogen, in the parts of the tissue which are most attacked, with a diminution in soluble nitrogen."

In other words the percentage ratio of protein nitrogen to total nitrogen is lower in the "relatively healthy" region than in either the growth or the normal tissue. A typical example of Robin's findings is as follows:

	$\frac{\text{Protein N}}{\text{Total N}} \times 100$
Most cancerous region	58.01
Relatively healthy region	47.01
Normal liver (after 26 hours' autolysis)	70.55

Robin considered that the most logical interpretation of these facts was that the proteins of a cancer are formed at the expense of the products of autolysis of the proteins of the organ in which it develops. This view implies that the tissue immediately surrounding the growth is abnormal with respect to autolysis, and the point is of such importance that it was considered worthy of further investigation.

In the present investigation nitrogen analyses were carried out using rabbit tissues—cancerous, adjacent normal tissue, and the normal tissue of the same organ.

EXPERIMENTAL.

The rabbit tissue used for the experiments was liver and kidney in which metastatic deposits of malignant growth were present, the rabbits having been

¹ This investigation was undertaken on behalf of the Liverpool Medical Research Organisation, Prof. W. Blair Bell, Director, University of Liverpool.

previously inoculated with the Brown-Pearce tumour. On removing the organ from the animal the growth was cut away from the rest of the organ and the adjacent normal tissue also removed from the rest of the normal tissue. A representative sample of each section was submitted to independent histological examination.

The tissue was first minced up as finely as possible with a pair of scissors and then weighed out in portions of 0.3–0.5 g. This is the smallest weight of tissue compatible with uniform samples. Six samples of each section of tissue were weighed out, two in combustion test-tubes for total nitrogen determinations, two in small glass tubes for protein nitrogen determinations and two in small tubes for dry weight estimations. The last two were heated at 105–115° for 36 hours in an electric oven. It was found that this drying was enough to give constant dry weights and the results could be reproduced to within 2 %.

Following the well-known micro-Kjeldahl technique [Pregl, 1924] for total nitrogen determination, there was added to each sample 1 cc. concentrated sulphuric acid, a knife-point of nitrogen-free copper sulphate and the same amount of potassium sulphate, and the mixture heated over a micro-burner until it charred. A few drops of 30 % hydrogen peroxide were then added and the mixture again heated to charring. This process was repeated until all the organic matter had disappeared, judging by the absence of charring. The time required is shortened considerably if before adding the sulphates the tissue is charred with concentrated sulphuric acid then heated for some time in a water-bath, a few drops of hydrogen peroxide being added from time to time. After the oxidation was complete the contents of the tube were diluted and made up to 25 cc. 2 cc. samples were taken and the nitrogen estimated in a micro-Kjeldahl distillation apparatus. Blank experiments were made with concentrated sulphuric acid, copper sulphate, potassium sulphate, distilled water and hydrogen peroxide.

Each sample for protein determination was thoroughly mixed with pure anhydrous sodium sulphate and allowed to harden. The hard mass was transferred to a small flask with absolute alcohol and boiled on a water-bath for an hour. The liquid was then centrifuged and the remaining mass extracted with boiling water slightly acidified with acetic acid. The extraction was continued until the extracting liquid showed no sign of sulphate. The remaining proteins were incinerated and the nitrogen estimated by the micro-Kjeldahl method as before.

Samples (in duplicate) were taken from tissue representing the three regions, *viz.* the growth, the adjacent normal tissue, and the normal tissue, and also duplicate distillations were carried out with each sample. The results obtained by the above method agree to within 2 %.

RESULTS.

In all, tissues from 16 rabbits were analysed and the results are summarised in Table I.

Table I. *Analytical results.*

Rabbit No.	6	7	11	12	13	4	5	8	2	1	10	14	15	16	3	9
Description of tissue	K.	L.	L.	L.	K.	K.	L.	L.	K.	K.	K.	K.	L.	K.	K.	K.
P.M. period (hours)	0	0	0	0	0	1	1	3	5	12	12	12	12	12	24	24
Growth																
Dry weight	18.9	21.4	21.8	22.3	20.4	15.5	22.7	21.3	21.2	20.5	18.1	22.0	24.8	20.6	20.3	20.9
Total nitrogen	2.68	2.86	2.79	3.05	2.79	2.83	3.36	2.92	3.26	2.75	2.13	3.26	3.67	2.84	2.70	2.69
Protein "	1.72	2.42	2.44	2.55	2.21	2.26	2.56	2.35	2.63	2.34	1.97	2.62	3.23	2.60	2.45	2.23
Soluble "	0.96	0.44	0.35	0.50	0.58	0.57	0.80	0.57	0.63	0.41	0.16	0.64	0.44	0.24	0.25	0.46
Adjacent normal tissue																
Dry weight	20.4	24.0	23.5	26.7	22.2	16.6	22.2	23.0	19.8	20.2	19.6	20.5	24.1	19.6	17.5	17.4
Total nitrogen	3.03	3.25	2.98	3.48	3.66	2.27	3.17	3.39	3.15	2.72	2.59	2.88	3.76	2.78	2.38	2.44
Protein "	2.27	2.90	2.70	3.00	3.24	1.79	2.45	2.89	2.45	2.02	2.22	2.20	2.97	2.30	2.10	1.96
Soluble "	0.76	0.35	0.28	0.48	0.42	0.48	0.72	0.50	0.70	0.70	0.37	0.68	0.79	0.48	0.28	0.48
Normal tissue																
Dry weight	19.9	—	22.6	25.2	21.8	15.9	23.5	25.1	18.6	—	15.9	22.9	24.2	18.2	15.9	17.9
Total nitrogen	2.74	—	2.56	3.73	3.44	2.25	3.08	3.51	2.81	2.80	1.96	2.85	3.68	2.30	2.14	2.41
Protein "	2.13	—	2.48	3.61	3.20	1.76	2.70	3.09	2.35	2.62	1.83	2.53	2.92	2.15	1.91	2.05
Soluble "	0.61	—	0.08	0.12	0.20	0.49	0.38	0.50	0.46	0.18	0.13	0.32	0.39	0.15	0.23	0.36
P.M. period																
Growth	0	0	0	0	0	1	1	3	5	12	12	12	12	12	24	24
Adjacent normal tissue	64.2	84.6	87.5	83.6	79.2	79.9	76.2	80.5	80.7	85.1	92.5	80.4	88.0	91.6	90.7	82.9
Normal tissue	67.3	89.2	90.6	86.2	88.5	78.9	77.3	85.3	77.8	74.3	85.7	76.4	79.0	82.7	88.2	80.3
	77.7	—	96.9	96.7	93.1	78.2	87.7	88.0	83.6	93.6	93.4	88.8	79.4	93.5	89.3	85.1

Percentage ratios of protein nitrogen to total nitrogen

In the second horizontal column the letters K. and L. represent kidney and liver respectively.

The *post mortem* period, represented by P.M. period, is the time elapsing between the death of the animal and the excision of the tissue.

The "dry weight" is the percentage ratio of the weight of dried tissue to the weight of the tissue before drying, *i.e.* fresh tissue.

The nitrogen figures represent, in all cases, the percentage weight of nitrogen in fresh, *i.e.* wet tissue.

At the foot of Table I are given the percentage ratios of protein nitrogen to total nitrogen in the growth, adjacent normal tissue and normal tissue.

DISCUSSION OF RESULTS.

In the first place it is necessary to draw attention to the conditions under which Robin's results were obtained. Robin worked with human liver, stomach and pancreas which were obtained *post mortem*. As a consequence the tissue was not excised from the corpse until 24–27 hours after death. As will be seen later this fact probably accounts for the results that Robin obtained. The normal tissue employed by Robin was tissue from a healthy person killed in an accident. In the present investigation the normal tissue was taken from the same organ as the growth and consequently probably gives a more useful comparison than that of Robin. It follows that Robin's results and the group with P.M. period of 12 hours in the present investigation ought to be comparable.

The results obtained by the writer indicate fundamental differences according to the P.M. period of the tissue employed. In the group with P.M. period less than 3 hours the percentage ratio of protein nitrogen to total nitrogen is greatest in the normal tissue, lowest in the growth, with an intermediate value in the adjacent normal tissue. When the P.M. period is 12 hours the ratio is lower in the adjacent normal tissue than in either the normal tissue or the growth—this might for convenience be known as the "Robin effect." When the P.M. period exceeds 12 hours this "Robin effect" seems to be less pronounced. Hence if the tissue is analysed when it is as near as possible to the condition obtaining *in vivo*, *i.e.* P.M. period less than 3 hours, there is no unexpected proteolytic condition in the adjacent normal tissue. The "Robin effect" manifests itself after a *post mortem* period of 12 hours for rabbit tissue. Hence this effect is probably due to *post mortem* changes and does not represent conditions of malignancy *in vivo*.

At first sight this minimum value of the ratio protein nitrogen to total nitrogen in the adjacent normal tissue after a P.M. period of 12 hours seems to be explained by assuming that autolysis takes place to the greatest extent in this region. This can be shown not to be the case by consideration of Table II. The second column gives the percentage values of protein nitrogen in the adjacent normal tissue, and the third column gives the percentage

values of protein nitrogen in the normal tissue calculated on the basis of the same dry weight as the corresponding adjacent normal tissue.

Table II.

Rabbit No.	Protein N of the adjacent normal tissue	Calc. protein N of the normal tissue	Rabbit No.	Protein N of the adjacent normal tissue	Calc. protein N of the normal tissue
2	2.45	2.50	10	2.22	2.26
3	2.10	2.10	11	2.70	2.58
4	1.79	1.84	12	(3.00)	(3.72)
5	2.45	2.55	13	3.24	3.21
6	2.27	2.18	14	2.20	2.26
8	2.89	2.83	15	2.97	2.91
9	1.96	1.99	16	2.30	2.32

It will be seen that in each case, whatever the P.M. period, the values in columns 2 and 3 are the same within experimental error. This shows that autolysis is not occurring at an excessive rate in the adjacent normal tissue. Incidentally the above figures show that the growth does not, *in vivo* or *post mortem*, liberate proteolytic enzymes into the adjacent tissue.

In an attempt to understand these *post mortem* changes the figures giving the concentration of soluble nitrogen in the tissues may also with advantage be classified according to the P.M. period of the tissue:

Table III. *Concentration of soluble nitrogen.*

(% N per weight of water in tissue.)

	Rabbit No.	P.M. period	Growth	Adjacent normal tissue	Normal tissue
Kidney	4	1	0.68	0.58	0.58
"	6	0	1.18	0.96	0.76
"	13	0	0.73	0.54	0.26
Liver	5	1	1.04	0.93	0.49
"	7	0	0.56	0.46	—
"	8	3	0.72	0.65	0.67
"	11	0	0.45	0.37	0.10
"	12	0	0.64	0.66	0.16
Kidney	2	5	0.80	0.87	0.57
"	1	12	0.52	0.88	—
"	10	12	0.20	0.46	0.16
"	14	12	0.82	0.86	0.42
"	16	12	0.15	0.48	0.24
Liver	15	12	0.59	1.04	1.00
Kidney	9	24	0.58	0.58	0.43
"	3	24	0.30	0.34	0.27

It will be noticed that when the P.M. period is less than 3 hours the concentration of soluble nitrogen in the growth tends to be greater than that in the adjacent normal tissue which in turn tends to be greater than in the normal tissue.

When the P.M. period is 12 hours the concentration of soluble nitrogen seems to reach a maximum in the adjacent normal tissue. This is also the case in Robin's experiments on human tissue 24–27 hours *post mortem*.

When the P.M. period is 24 hours there seems to be a tendency for the concentration of soluble nitrogen to be equal in all three tissue regions.

From these figures it might be assumed that *in vivo* the concentration of soluble nitrogen is highest in the growth, becoming lower in the adjacent tissue and lower still in tissue further away from the growth. Hence on death it is possible that some controlling factor, such as the degree of permeability of the membranes, is altered and the soluble nitrogen travels from the place of highest osmotic pressure, *i.e.* the growth, to lower osmotic pressure regions. Thus in process of time soluble nitrogen will be transferred from the growth in the first instance to the adjacent normal tissue. This will mean that in the growth the total nitrogen will be lower while the protein nitrogen remains the same, that is the ratio of protein nitrogen to total nitrogen will be higher than *in vivo*. Also the adjacent normal tissue will increase in total nitrogen while the protein nitrogen is unaltered, so that the ratio of protein nitrogen to total nitrogen will be lower than *in vivo*. This is the condition we have called the "Robin effect."

The maximum concentration of soluble nitrogen in the adjacent normal tissue after 12 hours *post mortem* is rather puzzling but it is possibly due to some property of the tissue membranes. After 24 hours the membranes may be regarded as having become completely permeable and the concentration of soluble nitrogen tends to be the same in each of the regions.

In Table I all the percentages are referred to fresh (wet) tissue. Additional evidence in support of *post mortem* diffusion causing the different effects is however afforded by an examination of the dry weights of the tissues. From Table IV it will be noticed that when the P.M. period is less than 3 hours the

Table IV. *Dry weights.*

	Rabbit No.	P.M. period	Normal tissue	Growth
Kidney	4	1	15.9	15.5
"	6	0	19.9	15.5
"	13	0	21.8	20.4
Liver	5	1	23.5	22.7
"	7	0	—	21.1
"	8	3	25.1	21.3
"	11	0	22.6	21.8
"	12	0	25.2	22.3
Kidney	2	5	18.6	21.2
"	10	12	15.9	18.1
"	1	12	—	20.5
"	14	12	22.9	22.0
"	16	12	18.2	20.6
Liver	15	12	24.2	24.8
Kidney	9	24	17.9	20.9
"	3	24	15.9	20.3

growth tends to contain more water than the normal tissue. This is the usual behaviour of rapidly growing tissues. However when the P.M. period is 12 hours and over, the position is reversed and the normal tissue tends to be greater in water content than the malignant region. Hence, after death, water

begins to disappear from the growth into the surrounding organ. This movement of water makes it very probable that soluble nitrogen is also transferred throughout the organ after death.

The evidence against the Robin view that the normal tissue adjacent to a malignant growth is abnormal *in vivo* in a proteolytic sense is the following:

(a) the histological examination of normal tissue and adjacent normal tissue does not indicate any difference between them;

(b) in both regions the protein nitrogen content is the same when calculated on the same basis with respect to water content;

(c) when the P.M. period is less than 3 hours the ratio of protein to total nitrogen in the adjacent normal tissue is intermediate between the values for the growth and the normal tissue and not below both of them.

SUMMARY.

Malignant, adjacent normal, and normal rabbit tissues have been analysed for total nitrogen, protein nitrogen and soluble nitrogen. The results obtained show that Robin's [1919] view that in malignancy, normal tissue is autolysed and that the growth builds up its proteins from the autolytic products is not correct. The "Robin effect" has been obtained by allowing the tissue to remain in the body for 12 hours after death before excision. This effect has been shown to be due probably to *post mortem* diffusion in the organ. The "Robin effect" does not manifest itself in the case of freshly examined tissue and is therefore not operative *in vivo*.

Evidence has been obtained to the effect that after death water disappears from the growth into the surrounding tissue.

Evidence has also been obtained that a malignant growth does not liberate proteolytic enzymes into the surrounding tissues.

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