THE CYTOTOXIC ACTION OF IMMUNE GAMMA GLOBULIN AND COMPLEMENT ON KREBS ASCITES TUMOR CELLS*

II. Chemical Studies

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When Krebs ascites tumor cells are treated *in vitro* with rabbit gamma globulin containing antibodies to these cells, there occurs a distinctive structural alteration in the cell membrane visible in thin section by electron microscopy (1), but there are no structural changes in the interior of the cell. Upon the subsequent addition of fresh normal rabbit serum (complement), there very rapidly ensues a destructive process within the cytoplasm, involving the endoplasmic reticulum, mitochondria, and cytoplasmic matrix. The cell membrane, though greatly expanded as the result of the swelling of the cell, remains structurally intact in so far as can be seen under the electron microscope, so that any loss of cytoplasmic material must occur without cell lysis.

In the present work the cell composition was analyzed during the course of the action of antibody and complement in order to determine to what extent various chemical components were involved in the destructive process.

Methods

The gamma globulin fractions, tumor cells, and incubation conditions were similar to those described previously (1).

The Krebs-2 ascites tumor was maintained in mice by repeated transfers to the peritoneal cavity. To prepare cells for experiments, the ascitic fluid was placed in 40 ml. of cold balanced salt solution (2) containing glucose 1.0 mg./ml. (BSS). The suspension was centrifuged in the cold (400 R.P.M. in the International refrigerated centrifuge) for 10 minutes and washed once in the same medium. The final suspension in BSS was adjusted to about 2×10^7 cells per ml. The incubations were carried out in 5 or 10 ml. volumetric flasks, with a gas phase of 5 per cent CO_2, 95 per cent O_2, in a rotary shaker water bath at a speed just sufficient to keep the cells evenly suspended in the medium. Aliquots of cell suspension were examined periodically through the incubation period under phase microscopy, using a phase hemocytometer chamber (American Optical Company, Buffalo.)

The separation from other cell constituents of cold trichloracetic acid (TCA)-soluble ribonucleotides, ribonucleic acid (RNA), and desoxyribonucleic acid (DNA) was performed according to Schmidt and Thannhauser (3) as illustrated in Fig. 1. The fractions obtained

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were analyzed for pentose by the orcinol method (4) and for desoxypentose by the diphenylamine method (4). (It is assumed that the color production in these experiments is due to the corresponding purine nucleotides.)

In considering the results, acid-insoluble ribonucleotides are referred to as RNA and the acid-insoluble desoxyribonucleotides, DNA. It should be noted however that these substances might be appreciably degraded from the native state, even though acid-insoluble. The acid-soluble fraction contains the free nucleotides of the cell, any free pentose (probably neglible in comparison) and the lower molecular weight breakdown products of the nucleic acids.

The effect of antibody + complement on cell amino acids and proteins was examined in separate experiments by the method illustrated in Fig. 2. Amino acids (in the TCA-soluble

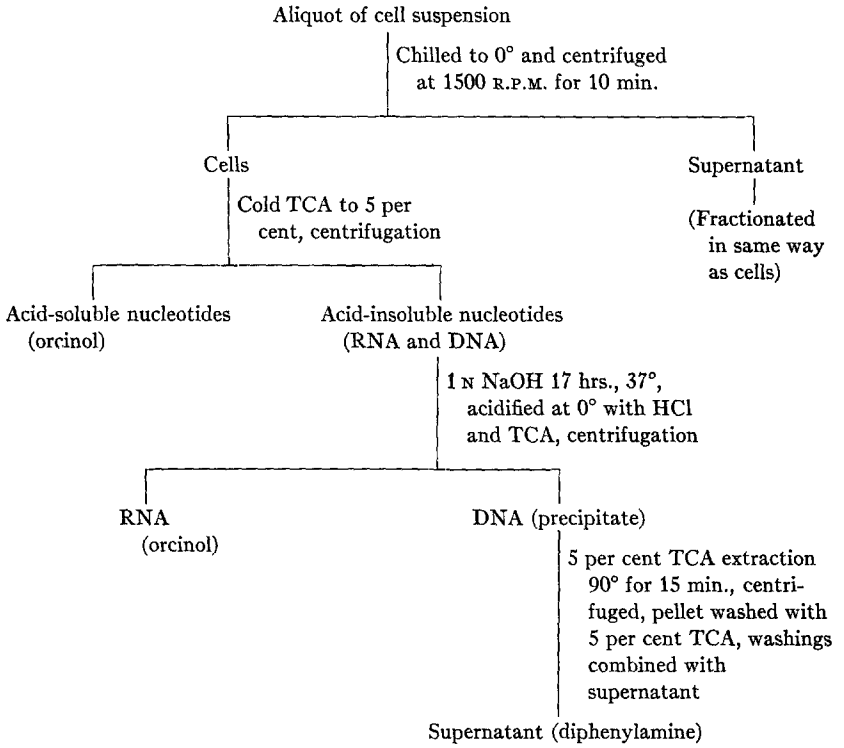

FIG. 1. Separation scheme for nucleotides and nucleic acids.

fractions) were determined by the Rosen modification of the ninhydrin method (5). This color reaction is not entirely specific for α-amino acids, some color being produced by peptides, urea, and ammonia. In the present experiments, the latter two seem unlikely to give appreciable contributions, and therefore color production was taken as a measure of free amino acids.

Protein was determined by the method of Lowry *et al.* (6).

RESULTS

Nucleic Acids and Nucleotides.—An experiment was performed to test the effect of rabbit immune gamma globulin and complement on the acid-soluble

nucleotides, RNA, and DNA of the cells. The design of the experiment is summarized in Table I, and the results in Table II.

Flask 1 which contained complement but no antibody, and flask 2, which contained antibody but no complement, underwent very little change through the course of the

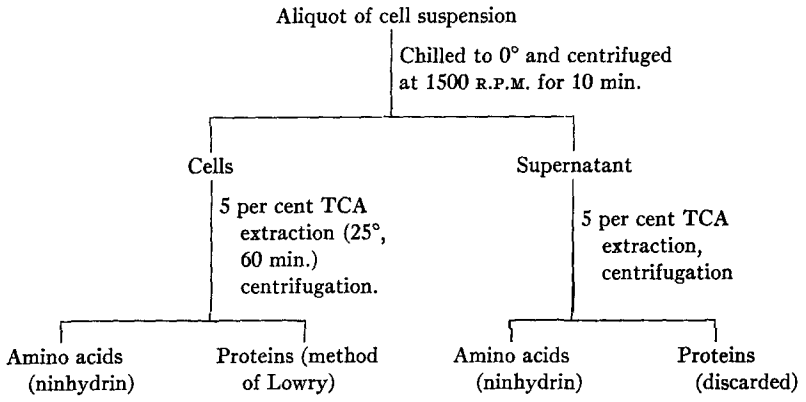

FIG. 2. Separation scheme for amino acids and proteins.

TABLE I

Nucleotides and Nucleic Acids

	Flask No.		
	1	2	3
	ml.	*ml.*	*ml.*
Rabbit anti-tumor cell gamma globulin (10 mg./ml.)	0	2.5	2.5
Mouse gamma globulin (10 mg./ml.)	2.5	0	0
Washed cell suspension (2×10^7 cells/ml.)	2.5	2.5	2.5
Fresh frozen normal rabbit serum (complement)	0.3	0	0.3
BSS	0	0.3	0

The reagents were added in the order shown and allowed to incubate for a few minutes before the final addition of complement, at time = 0. Incubation was continued for 60 minutes.

incubation.[1] There was, however, a slight increase in the acid-soluble nucleotides of the cells. In these flasks, the RNA content of the cells remained constant over the incubation period. The supernatant contained a small amount of RNA. This may

[1] The figures for acid-soluble pentose in the supernatant were quite high, owing to the presence in the incubation medium of 1.0 mg./ml. of glucose, which gave some color with the orcinol reagent. The values obtained were not corrected for the contribution of the glucose and they declined somewhat over the hour's incubation, presumably owing to consumption of the glucose by the cells. The other fractions did not contain appreciable quantities of contaminating glucose.

have been due to some cell injury in the course of preparation of the cells, or to incomplete sedimentation of the cells during centrifugation of the aliquots. In any case it remained constant through the incubation period.

In flask 3, containing immune gamma globulin and complement, by 10 minutes there was a marked loss in cell acid-soluble pentose and of RNA. At the same time

TABLE II

Nucleotides and Nucleic Acids

Flask No.	Time	μmoles pentose/10^9 cells					μmoles desoxypentose/10^9 cells
		Cells		Supernatant		Totals	Cells
		Acid-soluble pentose	RNA	Acid-soluble pentose	RNA		DNA
	min.						
1	0	5.75	54.0	10.6	5.20	75.6	29.8
(Control)	10	6.05	55.5	10.8	4.80	77.2	27.5
	30	7.10	—	8.05	—	—	—
	60	8.85	56.5	7.10	4.25	76.7	26.3
2	0	4.45	62.0	10.6	4.00	81.1	28.1
(Antibody)	10	5.02	55.5	7.85	4.00	72.4	26.5
	30	5.02	50.5	6.90	3.60	66.0	25.1
	60	6.95	52.0	6.90	4.80	70.7	24.9
3	0	4.45	62.0	10.6	4.25	80.3	27.5
(Antibody + C′)	10	2.07	35.5	32.8	3.82	73.2	28.7
	30	2.07	18.4	30.3	29.1	79.9	28.7
	60	2.07	14.4	28.2	27.3	72.0	28.0

1.0 ml. aliquots were withdrawn from each flask at intervals, and chilled rapidly in small tubes in an ice bath. The tubes were then spun for 10 minutes at 0° in the refrigerated centrifuge at 1500 R.P.M. The supernatant was decanted and the tube containing the cell pellet allowed to drain. The sedimented cells and an aliquot of the supernatant were put through the Schmidt-Thannhauser procedure and analyzed for TCA-soluble nucleotides, RNA and DNA.

Adenosinemonophosphate was the primary standard for the pentose determinations, and desoxyadenosine the primary standard for desoxypentose determinations.

there appeared in the medium an approximately equivalent quantity of acid-soluble pentose. By 30 minutes there was further loss of cell RNA, and appearance of RNA in the medium. In all, approximately three-quarters of the cell RNA was lost to the medium, part appearing in the form of acid-soluble, and the rest as acid-insoluble pentose. (The proportion of the RNA lost from the cells which appeared in the TCA-soluble fraction of the medium was variable in different experiments). There was little further change after 30 minutes' incubation. The total pentose values of cells + medium remained approximately the same for all flasks.

Antibody plus complement released no DNA from the cells into the medium. The sensitivity of the method was such that not more than 5 per cent of the total DNA could have passed into any of the other fractions, without detection. No acid-soluble desoxyribonucleotide was detectable in any fractions of control or treated cells.

It was of interest to know whether breakdown of RNA might result simply from the incubation of structurally disrupted cells, and therefore be only an indirect consequence of the action of antibody and complement. To test this point, cells were completely disrupted by sonic disintegration (Raytheon, Waltham, Massachusetts). The lysate was found to contain the same quantity of RNA as the intact cells, and simple incubation in BSS for 1 hour produced no decrease in RNA.

Free Amino Acids and Proteins.—The effects of antibody and complement on the free amino acid pool and on the total cell protein were examined in a very

TABLE III

Amino Acids and Proteins

	Flask 1	Flask 2	Flask 3	Flask 4
	ml.	*ml.*	*ml.*	*ml.*
Washed cell suspension (1.9×10^7 cells/ml.)	2.0	2.0	2.0	2.0
Normal mouse gamma globulin (10 mg./ml.)	0	2.0	0	0
Rabbit anti-tumor cell gamma globulin (10 mg./ml.)	0	0	2.0	2.0
BSS	2.5	0	0	0
Fresh rabbit serum (complement)	0	0.5	0	0.5
Heated rabbit serum (inactivated complement)	0	0	0.5	0

similar way. The experimental design (Table III) includes an extra control (flask 1). The results of an experiment are shown in Table IV.

In flasks 1 (no gamma globulin or complement), 2 (mouse gamma globulin + fresh complement), and 3 (rabbit immune gamma globulin + inactivated complement) there was very little change during the incubation period in the distribution of free amino acids between cells and supernatant. There is perhaps some decline in the amino acid content of the cells, and a slight increase in that of the supernatant, but in most cases this change amounts to less than 15 per cent. The total values (cells + supernatant) remained constant.

In flask 4, (containing immune gamma globulin + complement) there was within 10 minutes a loss of nearly two-thirds of the cellular amino acids. At the same time the amino acid concentration in the medium increased roughly threefold. This increase is somewhat more than can be explained by simple depletion to the medium of the free amino acid pool, for the total amino acid increased by about 35 per cent. There was no further change through the hour's incubation.

The precipitated proteins left after TCA treatment of the cell pellet were dissolved in 1 N NaOH, and the total protein measured. The results are shown in Table V.

It can be seen from these data that only in flask 4, containing the immune globulin and complement, were there any significant changes through the course

TABLE IV

Amino Acids

Amino acids in	Time	Flask 1 (Control)	Flask 2 (Control)	Flask 3 (Antibody)	Flask 4 (Antibody + C')
	min.	*μmoles*	*μmoles*	*μmoles*	*μmoles*
Cells	0	0.91	0.88	0.93	0.90
	10	0.87	0.88	0.87	0.39
	25	0.80	0.84	0.85	0.33
	60	0.87	0.86	0.78	0.35
Supernatant	0	0.37	0.43	0.39	0.43
	10	0.48	0.67	0.51	1.41
	25	0.46	0.54	0.40	1.42
	60	0.53	0.51	0.43	1.53
Total (cells + supernatant)	0	1.28	1.31	1.32	1.33
	10	1.35	1.55	1.38	1.80
	25	1.30	1.38	1.25	1.75
	60	1.40	1.37	1.21	1.85

1.0 ml. samples of cell suspension were taken at intervals, quickly chilled and centrifuged. To cells and aspirated supernatant were added TCA to a final concentration of 5 per cent, and the mixture was incubated for at least 90 minutes at room temperature. The precipitates were centrifuged and the amino acid concentrations of the supernatant measured by the ninhydrin method. The results are expressed as μmoles of amino acid in cells or in supernatant from 1.0 ml. of suspension (1.0×10^7 cells/ml.).

TABLE V

Proteins

Time	Flask 1 (control)	Flask 2 (control)	Flask 3 (antibody)	Flask 4 (antibody + C')
	Cell protein			
min.	*mg.*	*mg.*	*mg.*	*mg.*
0	2.00	2.26	2.47	2.47
10	2.16	2.52	2.58	2.19
25	2.40	2.40	2.61	1.89
60	2.40	2.55	2.58	1.62

of the incubation. In flask 4 the protein content of the centrifuged cells declined by about 30 per cent. In other experiments of a similar kind the total protein loss ranged from 30 to 60 per cent.

Uptake and Release of K^{42}.—The surface membrane change induced by anti-

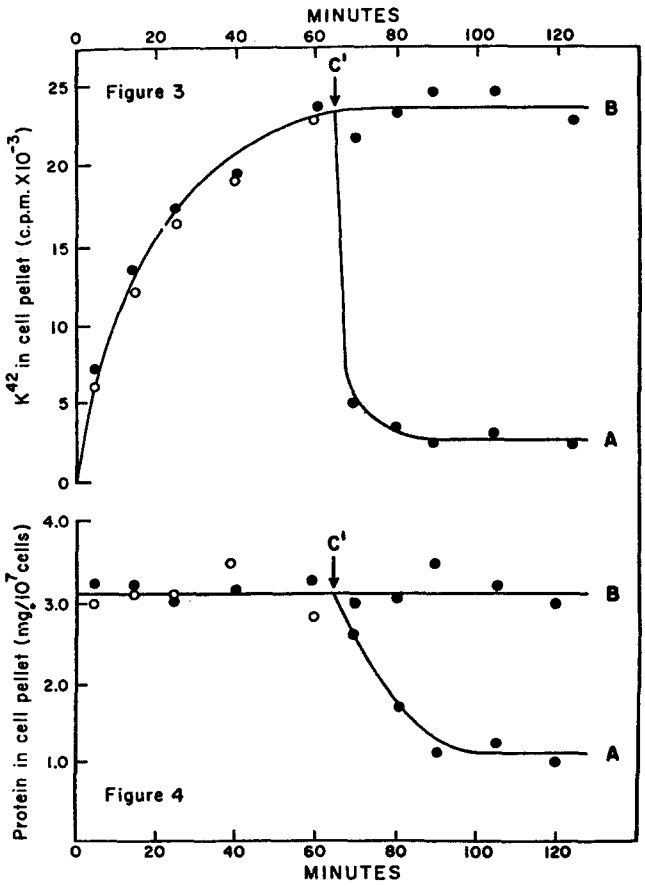

FIG. 3. Uptake and release of K^{42}.

FIG. 4. Loss of cell protein.

At time 0, 4.0 ml. of K^{42} in BSS was added to each of two 250 ml. flasks, whose contents were as follows:—

1. Indicated by ○
   - 26.0 ml. of cell suspension (2.0 × 10^7 cells/ml.)
   - 7.5 ml. of human serum albumin (10 mg./ml.)
   - 10.0 ml. of BSS (containing 50 mg. additional glucose)
2. Indicated by ●
   - 26.0 ml. of cell suspension (2.0 × 10^7 cells/ml.)
   - 7.5 ml. of rabbit immune gamma globulin (10 mg./ml.)
   - 10.0 ml. of BSS (containing 50 mg. additional glucose)

At time = 60 minutes, flask 1 was discarded. 15.0 ml. portions of the suspension in flask 2 were added to two 125 ml. flasks A and B.

At time = 65 min., 2.0 ml. of complement was added to A, and 2.0 ml. of inactivated complement to B.

body in the absence of complement (1) might be expected, even if it did not lead to net losses of cellular constituents, to influence the rate of exchange across the cell membrane of substances present both inside and outside the cell. It was therefore of interest to compare the rates at which K^{42} added to the incubation medium was taken up by cells in the presence and in the absence of immune globulin. The result of such an experiment is shown in Figs. 3 and 4.

A twice washed cell suspension was divided between two flasks; to one flask was added HSA, and to the other immune gamma globulin in the same concentration. At time = 0, K^{42} was added in tracer quantities, compared with the potassium concentration in the cells and incubation medium. At intervals thereafter, 3.0 ml. aliquots of the suspension were taken into tubes and immediately centrifuged at 1500 R.P.M. for 10 minutes in the refrigerated centrifuge. The supernatant was poured off and the pellets drained. The radioactivity remaining in the pellet was then counted in a gamma ray, well type scintillation counter.

Fig. 3 shows that there was no difference between the rates of uptake of the K^{42} in the presence of HSA and of immune gamma globulin. The rate of uptake was rapid at first, but within about 60 minutes, the same steady-state distribution was reached between the concentrations of K^{42} in cells and supernatant.

At time = 60 minutes, the cell suspension containing HSA was discarded and the cell suspension containing immune gamma globulin was divided between two flasks. At time = 65 minutes, fresh complement was added to flask A, and an equal volume of inactivated complement to flask B. Within 5 minutes (Fig. 3), the cells in flask A had lost the bulk of their potassium, and they lost only slightly more during the remainder of the incubation period. The cells in flask B lost no K^{42} whatever during the same interval.

The protein contents of the cell pellets of flasks A and B were also determined (Fig. 4). The cells of flask A lost protein, during the same interval as they lost K^{42}, but not at the same rate. Within the first 5 minutes after the addition of complement the cells lost only about 15 per cent of their protein, though they had lost about 75 per cent of their potassium. Over the rest of the incubation period, the cells lost progressively more of their protein, while the potassium curve quickly approached its maximum loss of 90 per cent. The more rapid rate of loss of potassium than of protein was a constant feature of these experiments.

DISCUSSION

It appears from the data that the visible effects produced in cells by the action of antibody and complement (1) are accompanied by the following biochemical changes:

1. Loss to the medium of a large fraction of the cell pool of free amino acids and ribonucleotides.

2. Loss to the medium of most of the cell RNA. Part of what is lost is probably appreciably degraded, since it appears in the acid-soluble fraction

of the supernatant. The rest though remaining acid-insoluble, might also be partly degraded.

A loss of cytoplasmic RNA from cells treated with antibody and complement was correctly inferred from a study of loss of staining properties of the cytoplasm (basophilia and pyroninophilia) by Kalfayan and Kidd (7) and Flax (8). The present study confirms and extends the data of Colter *et al.* (9) on the release of TCA-precipitable phosphate and of protein from a variety of cell types treated with antisera to cell ribonucleoprotein. While the latter study did not determine which of the effects observed required the presence of complement, it may be safely concluded on the basis of the results reported here that antibody alone would not have produced the results reported.

Some data on the appearance of the TCA-soluble phosphorus in the medium from Ehrlich ascites cells treated with antibody and complement have recently been reported by Ellem (10, 11). These results are, on the whole, consistent with those reported here for acid-soluble ribose, as both are presumably a measure of free nucleotides. The loss of nucleotides from cells treated with antibody alone in the experiments of Ellem are to be interpreted as the result of physical disruption of the cells by repeated centrifugation in his experimental procedure. It is likely that the membrane change and associated agglutination produced in the Krebs ascites tumor cells by antibody alone (1) occurred as well in the Ehrlich ascites tumor cells of Ellem's experiments. This probably is responsible for the greater sensitivity of the cells to physical disruption by the repeated centrifugation and resuspension.

Some idea as to the source of the lost and degraded RNA which comes from the cells may be gathered from the known intracellular distribution of RNA. In the similar Ehrlich ascites tumor cell, about 90 per cent of the cytoplasmic RNA is sedimentable in the ultracentrifuge (12). Since this RNA is concentrated largely in the RNA-rich granules of the endoplasmic reticulum and cytoplasmic matrix, the loss of two-thirds of the cell RNA in the present experiments must affect at least the larger of these two stores. The appearance of the cells in electron microscopy (1) suggests that the RNA granules of the cytoplasmic matrix may be most affected, and it is possible that they alone might be contributing the bulk of the RNA products appearing in the medium.

The cause of the apparent destruction of RNA observed here has not been elucidated. Such a breakdown could be due to the direct action of complement which, it has been shown, can acquire the properties of an esterase (13), or it could be due to activation of latent intracellular hydrolytic enzymes by the antigen-antibody-complement complex.

3. Loss of a large and variable fraction of the cell protein ranging between 30 and 60 per cent.

4. Increase (approximately 35 per cent) in total ninhydrin reacting material in the cell suspension exposed to antibody and complement. It is possible

that this is amino acid liberated from the hydrolytic breakdown of the cellular protein, or other substances, such as glutamine, or glutathione could conceivably be split and contribute to this increase. Since the amino acid pool represents only about 5 per cent of the total amino acid which could be liberated from cell protein by hydrolysis, a 35 per cent increase in the free amino acids could be supplied by hydrolytic degradation of only a very small proportion of the total cell protein. No attempt was made to measure, in the supernatant, protein released from the cells, since the amount of released protein was estimated to be too small to be detected in the presence of the high concentrations of immune gamma globulin and complement added to the medium.

5. Very rapid loss of up to 90 per cent of the intracellular potassium.

6. Absence of any loss of DNA from the cells, or any evidence of degradation. This is consistent with the substantially intact appearance of the cell nuclei in the electron microscope (1), and is in agreement with the results of Colter *et al.* (9).

It is noteworthy that in all the experiments reported here, concomitant observation of the cells by phase microscopy showed that even at the end of 60 minutes' incubation only an occasional cell was lysed. The electron microscopy of cells incubated in the same way confirms the absence of any visible disruption in the cell membrane (1), and others have made the same observation on a variety of cell types (7, 11). The loss of potassium, amino acids, nucleotides, RNA and proteins reported here therefore occurs through an unruptured cell membrane. Consistent with this, it is to be noted that the small molecules—potassium, amino acids, and nucleotides escape from the cell more rapidly than the macromolecules—RNA and protein. Evidently, the permeability of the cell membrane is not so increased as to completely remove selectivity with respect to molecular size of the products passing out.

SUMMARY

The *in vitro* exposure of Krebs ascites tumor cells to the action of rabbit immune gamma globulin alone does not result in any changes in the cell concentration of amino acids, ribonucleotides, RNA, DNA, or protein, nor in the rate of entry of potassium into the cell.

The exposure of the cells to antibody + complement results in the following changes within a few minutes:—

(*a*) Loss of about two-thirds of the free amino acids and ribonucleotides.

(*b*) Loss of about 90 per cent of the intracellular potassium.

(*c*) Loss of about three-quarters of the cell RNA to the medium, part appearing as TCA-soluble and the rest as TCA-insoluble products. There were no changes detectable in DNA.

(*d*) A small increase in total free amino acid of the cell suspension.

(*e*) Loss of from 30 to 60 per cent of the cell protein.

The loss of these substances is believed to occur through a cell membrane which is still intact, as judged by phase and electron microscopy, and still able to discriminate to a small degree against passage of larger molecules.

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