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INHIBITOR OF PROTEIN DEGRADATION FORMED DURING INCUBATION OF ISOLATED RAT HEPATOCYTES IN A CELL CULTURE MEDIUM

Its Identification as Ammonia

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SUMMARY

Isolated rat hepatocytes, incubated as a suspension in a tissue culture medium (Dulbecco's), are in negative nitrogen balance and exhibit a continuous net release of amino acids and/or urea. The cells also generate ammonia and/or urea by the deamination of medium glutamine. Under normoxic conditions, ammonia is rapidly converted to urea, but under hypoxic conditions, arising, e.g., if the cells are allowed to sediment, ammonia accumulates. By interference with the analytical procedures and the calculation of nitrogen balance, the accumulation of ammonia may give a false impression of cellular hyper-catabolic activity during the first hour of incubation. When this effect has subsided (or been corrected for), it becomes evident that the accumulated ammonia exerts an inhibitory influence on protein degradation, reducing both the total nitrogen release and the liberation of [^{14}C]valine from pre-labelled liver cell protein. The fact that protein degradation is inhibited by ammonia may be of considerable importance for the growth of cells under tissue culture conditions, where ammonia is so readily generated by the spontaneous or metabolic deamination of glutamine.

Isolated rat hepatocytes in suspension, although actively synthesising protein [1–5], degrade their endogenous protein at such a high rate (4%/h) as to be in a highly negative nitrogen balance [6–8]. Nevertheless, these cells are able to form monolayers and survive in culture for several days [9–11], indicating that the protein-catabolic state can be reversed by the culture conditions and/or changes in cellular properties.

In a search for factors which might improve the nitrogen balance of isolated hepatocytes, it was found that incubation of the cells for several hours in a tissue culture medium (Dulbecco's) appeared to reduce their loss of nitrogen strongly [8]. In the present paper, this effect of Dulbecco's medium is further investigated.

Some aspects of this work have been reported in a preliminary form [7].

METHODS

Isolated rat hepatocytes were prepared from the livers of 16 h fasted, 250–300 g male Wistar rats (maintained on an "8+16" feeding schedule, cf [12]) by the method of collagenase perfusion [13–15]. The hepatocytes were suspended in organically buffered saline and routinely incubated in rapidly shaking centrifuge tubes as previously detailed [15, 16]. For incubations of several hours' duration, slowly shaking Petri dishes were used. This is a gentle incubation method which preserves the structural integrity of the cells for at least 7–8 h [2, 15].

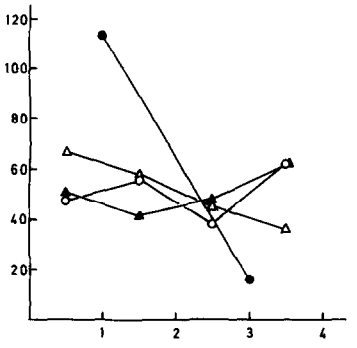

Fig. 1. Abscissa: incubation time (hours); *ordinate:* apparent rate of nitrogen release (μgatoms/g cells/h).

Effect of Dulbecco's medium on apparent nitrogen release from isolated rat hepatocytes. The cells were incubated for 4 h at 37°C in slowly shaking Petri dishes with either ●, Dulbecco's medium; ○, plain suspension buffer; ▲, suspension buffer containing 0.1 IU/ml of insulin; △, suspension buffer containing an amino acid mixture [5] at 5×normal concentration. The apparent rate of nitrogen release (i.e., the apparent rate of net formation of urea+amino acids) was calculated for each of the two consecutive 2 h periods in the case of Dulbecco's medium; and for each of the four consecutive 1 h periods in the case of the other incubates. Each value is the mean of two cell samples.

Total amino acids were determined by the ninhydrin method [17], and urea (or ammonia) with the Sigma urea nitrogen kit no. 14. The latter method is based on the conversion of urea to ammonia by urease treatment, and subsequent determination of ammonia with the Nessler reagent. The method can therefore be used for separate determination of ammonia or urea (±urease). However, under standard incubation conditions the amount of ammonia in the liver cell suspensions was very low (about 10% of the urea nitrogen content or less), and it was therefore considered unnecessary to distinguish between urea and ammonia, i.e., the analysis was performed so as to include both. Since the objective of these experiments was, furthermore, to assess the overall nitrogen balance, it was reasoned that a combined measurement of urea+ammonia would give the same value regardless of the relative contents of each of the two [8]. However, this assumption ignores the fact that ammonia also reacts with ninhydrin [17] with almost the same efficiency as the leucine standard (a colour factor of 0.92 for ammonia relative to leucine was experimentally determined); hence ammonia will be included both in the total amino acid analysis (ninhydrin method) and in the analysis of urea+ammonia. While the resulting overestimation of total acid-soluble nitrogen is negligible under normal conditions, it can cause a significant error if ammonia is present in appreciable amounts, as is the case in most of the experiments described in the present paper. For this reason, the measured values are referred to in figures and tables as "apparent" unless corrections for the ammonia content have been made.

The sum of the net amounts of amino acids, urea and ammonia formed by the cells is taken to represent the total nitrogen release; the term "release" referring to the transition from acid-insoluble (i.e., protein) to acid-soluble forms of nitrogen-containing molecules. Although metabolic analyses are performed on the complete system of cells+medium, the net release of the nitrogenous metabolites in question also corresponds to the release from the cells to the medium, since there is no retention of these metabolites intracellularly [5, 7]. All analytical values are expressed on a wet weight basis, i.e., per gram cellular weight [14].

For incubations in Dulbecco's modified Eagle's medium, a commercially available powdered medium (Gibco), dissolved in H_2O with the organic buffers HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid); TES (*N*-Tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid); and Tricine (*N*-Tris-(hydroxymethyl)methylglycine) rather than with bicarbonate, was used initially. Later, the medium was made in the laboratory by mixing the various groups of ingredients as described in table 2.

When a certain atmospheric O_2 concentration was desired during incubation, the centrifuge tubes used as incubation vessels were filled with the appropriate N_2/O_2-mixture and stoppered. The pH value was varied by addition of the appropriate amounts of NaOH or HCl (the amount determined by titration of a model incubate).

[^{14}C]Valine (>225 mCi/mmole, CFB 75) was obtained from Amersham. Soluble and protein-incorporated radioactivity was measured by liquid scintillation counting as previously described [5].

Biochemicals were purchased from Sigma; other chemicals were of analytical grade.

RESULTS AND DISCUSSION

Time course of nitrogen release in Dulbecco's medium

In a previous communication [8] it was reported that rat hepatocytes in suspension degrade their endogenous protein at a rate of about 4%/h, as detected by a continuous release of nitrogen in the form of urea and amino acids. However, upon incubation in Dulbecco's medium in slowly shaking Petri dishes, a biphasic pattern of nitrogen metabolism was observed: a hyper-catabolic response (i.e., an apparent increase in nitrogen release) during the first 2 h period of incubation, followed by an anti-catabolic response (i.e., a strongly reduced nitrogen release) in the second 2 h period [8]. This phenomenon is depicted in fig. 1, and can

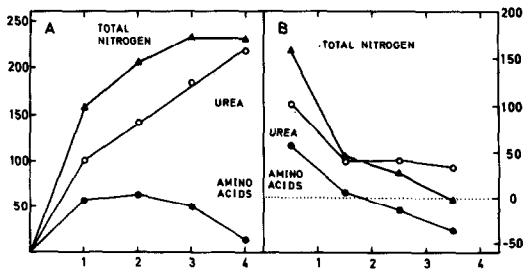

Fig. 2. Abscissa: incubation time (hours); *ordinate (left)* apparent nitrogen release (μgatoms/g cells); *(right)* apparent rate of nitrogen release (μgatoms/g cells/h).

Time course of apparent nitrogen release in Dulbecco's medium. Isolated rat hepatocytes in Dulbecco's medium were incubated for 4 h at 37°C in slowly shaking Petri dishes, and samples were taken each hour for determination of ●, amino acids; ○, urea; ▲, total nitrogen. (*A*) Total amounts accumulated; (*B*) rates of release for each of the consecutive 1 h periods. Values are expressed in terms of nitrogen equivalents (μgatoms); each value is the mean of four cell samples. Negative release rates (i.e., below dotted zero line) signify net consumption.

be seen to be characteristic for Dulbecco's medium. Control incubations with buffered saline or insulin showed a reasonably constant rate of nitrogen release throughout the 4 h period, whereas incubation with an amino acid mixture indicated a certain decrease in the catabolic rate, but much less pronounced than with Dulbecco's medium.

In fig. 2*A*, the time course of nitrogen release in Dulbecco's medium during a 4 h incubation in slowly shaking Petri dishes is shown. There was net accumulation of urea (including any ammonia) throughout the period, at a particularly high rate during the 1st hour, whereas the initial release of amino acids gradually changed into net amino acid consumption. The net result was an apparently very high rate of total nitrogen release during the 1st hour (the 1st hour response), followed by a precipitous decline detectable already in the 2nd hour (the 2nd hour response). Within 4 h, a state of practically zero nitrogen balance was established (fig. 2*B*).

Mixing of fresh and pre-incubated (conditioned) media and cells

In order to see whether the apparent decline in protein-catabolic rate (2nd hour response) was due to changes in the medium (i.e., medium-conditioning) or the cells, a mixing experiment was conducted. From incubated Petri dishes, medium and cells were separately removed at various intervals and reincubated for 1 h in rapidly shaking centrifuge tubes with fresh cells and medium respectively. As shown in table 1, fresh cells incubated with conditioned medium (B) immediately exhibited the same low catabolic rate as was observed in the Petri dishes after the 1st hour of incubation (A). On the other hand, cells preincubated in Petri dishes for up to 3 h immediately returned to a high protein-catabolic activity when reincubated with fresh medium (C). Clearly, the apparent decrease in catabolic rate observed during incubation in Dulbecco's medium reflected a change in medium composition rather than a change in the properties of the cells.

In the experiment reported in table 1, the "fresh" cells used for the testing of conditioned medium were actually stored on ice for various lengths of time (corresponding to the duration of medium preincubation). However, such storage at 0°C for up to 3–4 h did not diminish the protein-catabolic ability of the cells, as shown in table 1, col. D.

A striking methodological feature revealed by the experiment in table 1 was the disappearance of the 1st hour response (i.e., the apparent increase in catabolic rate) when cells were incubated in rapidly shaking centrifuge tubes (B–D, 0 h preincubation) rather than in slowly shaking Petri dishes (A, 0 h preincubation). Closer inspection indicated that incubation in Petri dishes tended to cause a partial sedimenta-

Table 1. *Effect of conditioned medium on nitrogen loss from isolated rat hepatocytes*

The cells were pre-incubated in Dulbecco's medium in slowly shaking Petri dishes at 37°C, or stored on ice. After various time lengths of pre-incubation, cells were separated from the medium (by centrifugation) and washed, and pre-incubated media or cells were re-incubated for 1 h in rapidly shaking centrifuge tubes together with fresh (ice-stored) cells (B) or medium (C) respectively. Some Petri dishes were re-incubated undisturbed (i.e., without separation of cells and medium) for an additional hour (A). In order to check that storage on ice did not affect the protein-catabolic state of the hepatocytes, cell samples were taken after various periods of ice-storage and incubated with fresh medium for 1 h in rapidly shaking centrifuge tubes (D). The nitrogen balance was calculated as described in Methods. Each value is the mean of two cell samples

	Apparent nitrogen release during re-incubation (μgatoms/g/h)			
Hours of pre-incubation (in slowly shaking petri dishes, A–C) or storage on ice (D)	A Re-incubated for 1 h in slowly shaking Petri dishes (no change of cells or medium)	Re-incubated for 1 h in rapidly shaking centrifuge tubes		
		B Pre-incubated medium+fresh (ice-stored) cells	C Pre-incubated cells+fresh medium	D Fresh (ice-stored) cells+fresh medium
0	118.4	64.3	80.4	68.9
1	24.3	15.2	54.9	77.2
2	34.4	17.9	64.0	73.9
3	22.0	16.1	66.6	69.8

tion of the cells. Since such sedimentation might possibly result in localized hypoxia [18], pH changes, etc., it was decided to study these parameters systematically by the use of centrifuge tube incubates, where controlled conditions can more easily be achieved.

Effects of pH and oxygen

Varying the pH in the range 6.6–8.0 had little effect on the overall nitrogen balance of cells incubated in Dulbecco's medium in rapidly shaking centrifuge tubes (fig. 3). Although a slight increase in the rate of urea formation with pH during the 2nd hour of incubation yielded a corresponding slight increase in the overall nitrogen loss, the general impression is that protein catabolism is relatively pH-insensitive. The transition from amino acid consumption during the 1st hour to amino acid release during the 2nd hour was balanced by a reduction in the rate of urea synthesis, resulting in very similar rates of overall nitrogen loss in the 1st hour period and the 2nd hour period (fig. 3). These changes in amino acid metab-

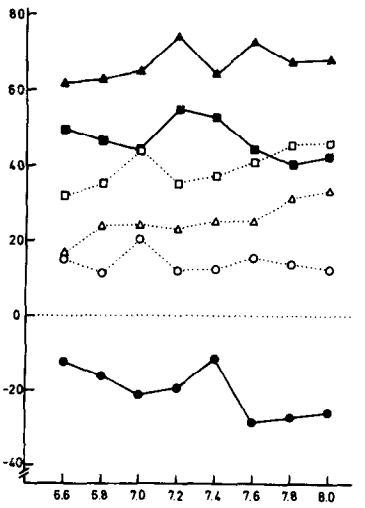

Fig. 3. Abscissa: pH; *ordinate:* apparent rate of nitrogen release (μgatoms/g cells/h).

Effect of pH on apparent nitrogen release in Dulbecco's medium. Isolated hepatocytes in Dulbecco's medium were incubated for 2 h at 37°C in rapidly shaking centrifuge tubes at different pH values. Medium pH was adjusted to the desired value by the addition of HCl or NaOH. Samples were taken at 0, 1, and 2 h, and the apparent rates of release of ●, ○, amino acids; ▲, △, urea; and ■, □, total nitrogen; were calculated for the 1st hour (●, ▲, ■—) and the 2nd hour (○, △, □ · · ·) periods respectively. Values are expressed in terms of nitrogen equivalents (μgatoms); each value is the mean of two cell samples. Negative values (below horizontal dotted zero line) signify net consumption.

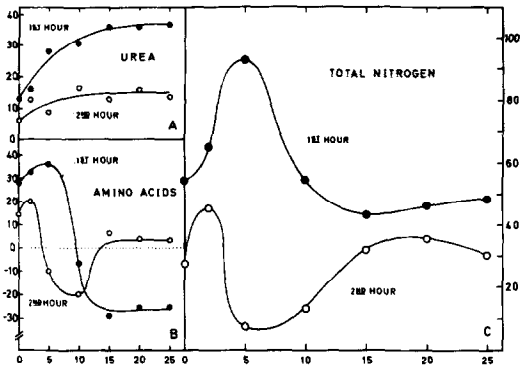

Fig. 4. Abscissa: atmospheric O_2 conc. (%); *ordinate* apparent rate of (*A*) urea formation; (*B*) amino acid formation; (*C*) total nitrogen release (μgatoms/g cells/h).

Effect of oxygen on apparent nitrogen release in Dulbecco's medium. Isolated hepatocytes were incubated in Dulbecco's medium for 2 h at 37°C in rapidly shaking centrifuge tubes. The stoppered tubes were filled with an O_2/N_2-mixture to give the O_2 concentration indicated. Samples were taken at 0, 1, and 2 h, and the apparent rates of release of (*A*) urea; (*B*) amino acids; and (*C*) total nitrogen were estimated for the ●, 1st hour; ○, 2nd hour periods, respectively. Each value is the mean of two cell samples; negative values (below dotted zero line in (*B*)) signify net consumption.

olism contrast with the situation in Petri dishes, where amino acid formation was maximal during the 1st hour, thus accounting for the 1st hour response (fig. 2).

In the experiment shown in fig. 4, the O_2 concentration was varied by incubating hepatocytes (in Dulbecco's medium) in stoppered tubes under an N_2/O_2-mixture of the composition indicated. As can be seen, oxygen had a dramatic influence on the pattern of nitrogen metabolism. Whereas the rate of urea formation increased as a function of the O_2 concentration both in the 1st and in the 2nd hour of incubation, amino acid metabolism varied with the O_2 concentration in a more complex way. During the 1st hour there was an apparent net release of amino acids at O_2 concentrations below 10%, and an apparent net consumption of amino acids above 10% O_2. This resulted in a pronounced peak of apparent catabolic activity at 5% O_2. Thus, the hyper-catabolic 1st hour response seen during incubation in slowly shaking Petri dishes (fig. 1, table 1) is most probably a result of the hypoxic conditions known to be generated in cellular sediments [18].

During the 2nd hour of incubation, amino acids were released both under normoxic (20% O_2) and completely anoxic (0% O_2) conditions; however, under hypoxic conditions (5–10% O_2) there appeared to be con-

Table 2. *Testing of components in Dulbecco's medium for their effect on the nitrogen balance of isolated rat hepatocytes*

The medium components of Dulbecco's medium (cf, e.g., any GIBCo catalogue) were divided into five groups to be tested separately and in combinations. The amino acids (group A) were dissolved at 100× concentration in 0.5 M NaOH (tyrosine and tryptophan first), while the majority of the vitamins (group B) were dissolved at 100× concentration in H_2O. The water-insoluble vitamins folate and riboflavin (group C) were dissolved at 100× concentration in 2 mM NaOH, and the unstable substrates glutamine and pyruvate were dissolved together with glucose at 16× concentration in fortified suspension buffer (group D). The CO_2/bicarbonate buffer used in the original Dulbecco's medium formulation was replaced by suspension buffer [14], a balanced salt solution containing the organic buffers HEPES, TES and Tricine. The suspension buffer was for this purpose fortified with additional Ca^{2+} and Mg^{2+} (total conc., $CaCl_2 \times 2H_2O$, 288 mg/l; $MgCl_2 \times 6H_2O$, 208 mg/l) to achieve approximately the same ionic composition as in the original Dulbecco's medium, while Fe^{3+} was omitted. The cells were incubated for 2 h at 37°C in rapidly shaking centrifuge tubes under an hypoxic atmosphere (5% O_2/95% N_2). Samples were taken after 1 and 2 h, and the net nitrogen balance each hour was calculated as described in Methods. Each value is the mean of two cell samples

Medium components added				Apparent nitrogen release (μgatoms/g/h)	
A	B	C	D	1st hour	2nd hour
●	–	–	–	61.9	21.0
–	●	–	–	47.9	43.1
●	●	●	–	70.5	34.1
●	●	●	●	168.6	38.0
●	●	–	●	156.5	26.7
●	–	●	●	150.4	22.4
–	●	●	●	147.2	23.4
–	–	–	●	136.8	45.2

sumption of amino acids. This, together with the low rate of urea formation, resulted in a minimal rate of total nitrogen release at 5% O_2. Thus, the anti-catabolic 2nd hour response observed during incubation of hepatocytes in Dulbecco's medium in slowly shaking Petri dishes (fig. 1, table 1) can also be explained as a result of hypoxia.

When the two curves for total apparent nitrogen release as a function of O_2 concentration are compared (fig. 4), the 1st hour response and the 2nd hour response can be seen to be virtually mirror images. This might suggest that the anti-catabolic 2nd hour response could be a consequence of the hyper-catabolic 1st hour response. Incubation of hepatocytes in Dulbecco's medium under hypoxic conditions might elicit a particular metabolic response (detectable as the 1st hour response), resulting in the accumulation of an anti-catabolic factor in the medium. During the 2nd hour of incubation, this factor would be present in sufficient amounts to depress the catabolic activity of the cells (2nd hour response), regardless of the O_2 concentration (nitrogen release is inhibited by conditioned medium even in well-aerated incubates, cf. table 1).

Search for the anti-catabolic factor in Dulbecco's medium

Since well-developed 1st and 2nd hour responses could be obtained by incubating hepatocytes with Dulbecco's medium in rapidly shaking centrifuge tubes under hypoxic conditions (5% O_2 in stoppered tubes), this system was used in a search for the medium component(s) responsible for eliciting these responses. For this purpose, the components of Dulbecco's medium were arranged in five different groups (table 2). The salts and buffers were common to all incubates, while the other groups were added in various combinations as shown in table 2. While the catabolic activity was generally lower in the 2nd than in the 1st hour of hypoxic incubation in this experiment, a pronounced hypercatabolic 1st hour response was only seen in those incubates containing group D substances (glucose, pyruvate and glutamine).

A second experiment, in which the three substances of group D were tested separately and in combinations (table 3), revealed that glutamine, which is present in large amounts in Dulbecco's medium, was the active component. In all combinations containing glutamine, both a strong hypercatabolic 1st hour response and a marked anti-catabolic 2nd hour response could be seen.

Liver cells are known to metabolize glutamine rapidly [19], and the observed responses could therefore very likely be a consequence of glutamine metabolism. Among the metabolites known to accumulate when glutamine is metabolized, glutamate, alanine, urea and ammonia (as NH_4Cl) were tested for their effect on overall nitrogen balance. Although there was a certain effect of alanine, a much more dramatic reduction of the apparent nitrogen loss was obtained when ammonium chloride was added (fig. 5). Therefore, the 2nd hour response could most likely be ascribed to ammonia, which might accumulate as the result of glutamine metabolism during the 1st hour of hypoxic incubation.

Formation of ammonia by glutamine deamination

The latter assumption was tested directly by incubating hepatocytes with 5 mM glutamine under normoxic (air) and hypoxic (5% O_2) conditions, and measuring ammonia and urea separately (i.e. ±urease in the assay). As shown in fig. 6, cells incubated with glutamine under normoxic conditions had an elevated rate of urea synthesis dur-

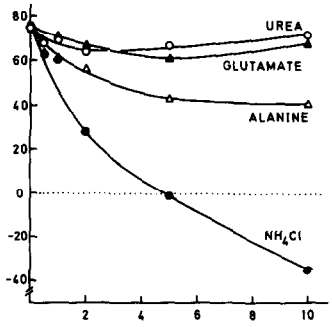

Fig. 5. Abscissa: initial conc. (mM); *ordinate;* apparent rate of nitrogen release (μgatoms/g cells/h).

Effect of various glutamine metabolites on the apparent nitrogen release from isolated hepatocytes. The cells were incubated in suspension buffer for 1 h at 37°C in rapidly shaking centrifuge tubes under an atmosphere of air, with the addition of ●, NH_4Cl; ○, urea; ▲, glutamate; or △, alanine at the initial concentration indicated. The apparent rate of nitrogen release during the incubation period was calculated on the basis of the measured changes in amino acids and urea+ammonia. Each value is the mean of two cell samples.

ing the 1st hour of incubation, indicating active degradation of glutamine. In the 2nd hour, ureogenesis had returned to control levels, presumably because most of the glutamine had been consumed. Under hypoxic conditions, however, the rate of urea formation was low even in the presence of glutamine (fig. 6*A*), whereas the rate of ammonia formation was greatly elevated (fig. 6*B*). Although a substantial accumulation of ammonia could be seen during the first 30 min after glutamine addition even under normoxia (fig. 6*B*), from 60 min onwards the concentration of ammonia was negligible unless hypoxic conditions prevailed. Apparently glutamine was rapidly deaminated at both oxygen concentrations, but under hypoxia the energy-requiring conversion of ammonia to urea became retarded, resulting in the accumulation of ammonia.

Interference by ammonia with analytical methods

At this point, two serious flaws in the basic assumptions underlying this investigation became apparent, both stemming from an underestimation of the importance of ammonia (assumed not to be present in significant amounts, which is usually the case under normoxic conditions). (1) The zero time values for the amino acid analyses, using the ninhydrin method, were found to be elevated proportionately to the amount of ammonium chloride added, demonstrating the known fact that ammonia reacts with ninhydrin [17]. Experimentally, a colour factor of 0.92 was found for NH_3 vs leucine in the ninhydrin assay, i.e., in addition to being included as a half-molecule of urea in the urea analysis, one molecule of ammonia would be scored as practically one molecule of amino acid in the amino acid analysis. Thus, in the computation of total nitrogen balance, any change reflecting alterations in the amount of ammonia would be exaggerated by a factor of two.

(2) One molecule of glutamine will give a ninhydrin reaction corresponding to one molecule of amino acid. However, deami-

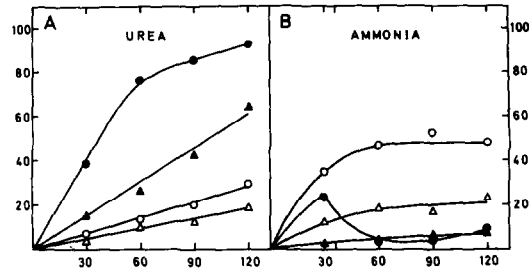

Fig. 6. Abscissa: incubation time (min); *ordinate:* (*A*) urea; (*B*) ammonia (μmoles/g cells).

Formation of urea and ammonia from glutamine under normoxic and hypoxic conditions. Isolated hepatocytes were incubated at 37°C in suspension buffer ▲, △, without or ●, ○, with 5 mM glutamine for the time indicated. Incubation took place in rapidly shaking, stoppered centrifuge tubes, either under normoxic conditions, i.e. in ●, ▲, an atmosphere of air, or ○, △, under hypoxic conditions, i.e. with 5% O_2/95% N_2. The accumulation of (*A*) urea, and (*B*) ammonia was measured at various time points. Each value is the mean of two cell samples.

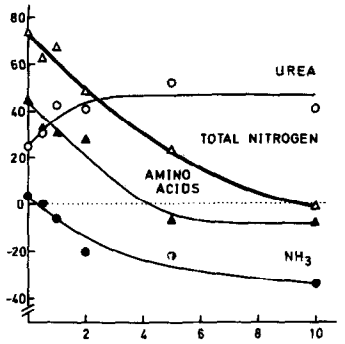

Fig. 7. Abscissa: initial NH_4Cl conc. (mM); *ordinate:* rate of nitrogen release (μgatoms/g cells/h).

Effect of ammonia on nitrogen release from isolated rat hepatocytes. The cells were incubated at 37°C for 1 h in rapidly shaking centrifuge tubes in the presence of NH_4Cl at the initial concentration indicated, and the rates of release of ▲, amino acids; O, urea; ●, ammonia; and △, total nitrogen during the incubation period were measured. The amino acid values were corrected for interference by ammonia. Each value is the mean of two cell samples; negative values (below dotted zero line) signify net consumption.

nation of glutamine produces two ninhydrin-positive molecules—glutamate and ammonia—and in addition, ammonia is included in the standard assay for urea (which routinely measures urea plus ammonia). By our analytical procedures, deamination of glutamine therefore results in the measurement of two extra nitrogen equivalents per molecule deaminated, and hence a large apparent nitrogen release is detected, falsely assumed to reflect protein catabolism.

The "hyper-catabolic" 1st hour response seen in Dulbecco's medium under hypoxic conditions can thus be fully explained as an accumulation of ammonia derived from the deamination of glutamine, and the phenomenon consequently is no reflection of cellular nitrogen balance.

Inhibition of protein degradation by ammonia

The anti-catabolic 2nd hour response, on the other hand, cannot be accounted for by the above considerations (i.e., by assuming re-synthesis of glutamine from glutamate and ammonia). In the experiment shown in fig. 7, ammonium chloride was added to cell incubates at increasing concentrations, and amino acids, urea and ammonia were separately analysed and corrected for ammonia interference. It can be seen that although the rates of ammonia consumption and urea formation (from ammonia) increase with the NH_4Cl concentration as expected, there is a decrease in the rate of amino acid formation and overall nitrogen release. Therefore, the anti-catabolic effect of ammonia indicated in fig. 5 persists also when the due corrections have been made. (In a short communication previously published [6], the amino acid values were properly corrected for interference by ammonia.)

It should be clear from the preceding discussion that any flow of ammonia nitrogen

Table 3. *Effect of glucose, pyruvate and glutamine on the nitrogen balance of isolated rat hepatocytes*

Cell suspension were made up in standard suspension buffer [14]. The cells were incubated at 37°C in rapidly shaking centrifuge tubes under an hypoxic atmosphere (5% O_2/95% N_2) in the presence of glucose (1000 mg/l), pyruvate (100 mg/l) or glutamine (600 mg/l) in various combinations. Samples were taken after 1 and 2 h, and the net nitrogen balance each hour was calculated as described in Methods. Each value is the mean of two cell samples

Substances added			Apparent nitrogen release (μgatoms/g cells/h)	
Glucose	Pyruvate	Glutamine	1st hour	2nd hour
–	–	–	52.8	35.1
●	–	–	51.4	46.0
–	●	–	50.1	51.1
●	●	–	53.2	51.4
●	●	●	135.0	−12.2
●	–	●	122.8	− 1.7
–	●	●	126.1	10.8
–	–	●	122.6	25.6

into ninhydrin-negative compounds (or sites) other than protein would produce a false "anti-catabolic" response. In order to verify the ammonia effect in an independent—and more direct—manner, the release of radioactivity from pre-labelled protein was measured. In the experiment shown in table 4, protein was labelled by the incorporation of [^{14}C]valine— an amino acid which is not metabolized by hepatocytes [20]—during a 1 h preincubation of the cells at 37°C. After washing, the cells were re-incubated in isotope-free medium, and the release of acid-soluble [^{14}C]valine from labelled protein was measured in the presence and absence of 10 mM NH_4Cl. As can be seen, ammonium chloride very significantly (42–43%) inhibited protein degradation as measured both by the disappearance of acid-insoluble radioactivity and by the accumulation of acid-soluble radioactivity (table 4). It would therefore seem safe to conclude that ammonia is an inhibitor of hepatocytic protein degradation, and that the anti-catabolic 2nd hour response observed after hypoxic incubation in Dulbecco's medium is due—at least in part—to the high concentration of ammonia present at that time.

General effects of glutamine and ammonia in tissue culture

Glutamine is an important component of most cell and tissue culture media, and a number of cultured cell lines have been shown to require high concentrations of this amino acid [21]. However, glutamine is not generally considered to be an essential amino acid, and it can in some cases be replaced by high concentrations of alanine or glutamate [22–24]. It is possible therefore that the role of glutamine in tissue culture is not primarily to be a substrate for protein synthesis, but rather to supply amino nitrogen for synthetic processes in intermediary metabolism.

Since glutamine under tissue culture conditions can be rapidly degraded both by spontaneous decomposition and by cellular metabolism [23, 25], accumulation of ammonia in the culture medium may be a general phenomenon which could possibly have some influence on the nitrogen balance and hence on the growth rate of cells. The generation of ammonia would be an aspect of "medium conditioning" worthy of consideration, since recent work has shown that cells as different as hepatocytes and macrophages are both sensitive to the anti-catabolic effect of ammonia (T. Berg, unpublished observations). Ammonia may therefore be a general inhibitor of protein degradation in all cells, a possibility which is strengthened by its mechanism of action. According to recent morphological and biochemical studies [26, 27], ammonia is a lysosomotropic agent which, because of its properties as a weak base, accumulates in the acidic interior of lysosomes. The resultant neutralization and swelling of the lysosomes reduces their proteolytic activity.

Whether ammonia can be regarded as a physiological feedback-inhibitor of protein degradation [7] is an open question, but at least the experiments reported in this paper make it clear that the anti-proteolytic effect of ammonia can come into play as the result of endogenous ammonia generation by cells maintained under near-physiological experimental conditions. Although rat hepatocytes require an hypoxic environment in order to prevent the further metabolization of ammonia to urea, other cells, which are not equipped with the urea cycle enzymes, should not be subject to this limitation. In spite of its in vivo toxicity (mostly neurotoxic symptoms, cf [28]) am-

Table 4. *Effect of ammonia on the release of acid-soluble radioactivity from pre-labelled protein*

Isolated rat hepatocytes were incubated for 1 h at 37°C in suspension buffer [14] with [^{14}C]valine (3 μCi/ml), then washed 7× with warm (37°C) washing buffer [14]. The washed cells were re-incubated for 2 h at 37°C in suspension buffer containing 1 mM cycloheximide, with or without 10 mM NH_4Cl. Samples were taken at 0 and 2 h, and the radioactivity was measured both in PCA precipitates (washed 3×) and extracts. Results are expressed as the amount of radioactivity lost from the precipitates, or appearing as acid-soluble counts, respectively. Values given are means ±S.E. of the number of cell samples indicated in parentheses. Significances were calculated by the *t*-test

	cpm/mg cells/h		% Inhibition by NH_4Cl	Significance of NH_4Cl effect
	Control	NH_4Cl (10 mM)		
Loss of acid-insoluble radioactivity (protein)	767±32 [9]	446±45 [9]	42	$P<0.001$
Accumulation of acid-soluble radioactivity (free valine)	612± 7 [19]	348± 5 [19]	43	$P<0.001$

monia appears to be relatively harmless to cells in culture. Rat hepatocyte monolayers incubated with 10 mM ammonium acetate for several days develop intensive lysosomal vacuolization, but their viability is undiminished, and levels of the steroid-metabolizing enzymes are better maintained in the presence of ammonia (J.-Å. Gustafsson & Å. Stenberg, unpublished observations). Unfortunately, rat hepatocytes are not capable of net growth under ordinary culture conditions [9–11], and it therefore remains to be shown whether the nitrogen-preserving action of ammonia could eventually result in increased growth. Most probably, the improved nitrogen status would be counterbalanced by the need for normal lysosomal functioning during growth [29].

Some specific effects of ammonia and glutamine

Ammonia or glutamine may have important effects in cell culture other than the inhibition of protein degradation. It has been shown that ammonia inhibits protein secretion in isolated hepatocytes, apparently by causing a swelling of secretory vesicles similar to that observed with lysosomes [30]. Protein synthesis on the other hand, appears to be relatively unaffected by ammonia [6, 30]. In isolated thyroid cells, ammonia inhibited iodide uptake [31]. Ammonia has a variety of effects on intermediary metabolism [32–34], and glutamine was found to be an essential amino group donor for the synthesis of complex glycosamine-containing surface carbohydrates by teratoma cells in culture [35].

Lectin-induced lymphoblastogenesis was reported to be inhibited in a tissue culture medium, and the inhibitor was identified as ammonia, generated by the deamination of glutamine [36]. The toxic action of diphtheria toxin on mouse macrophages was inhibited by ammonium chloride [37], and in slime mold differentiation ammonia was capable of exerting a directive influence [38]. While the mechanism of action of ammonia in these cases remains unknown, the examples serve to emphasize the importance of ammonia as a regulator of cellular function.

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