XLVI. STUDIES IN THE METABOLISM OF TISSUES GROWING *IN VITRO*.

I. AMMONIA AND UREA PRODUCTION BY KIDNEY.

By BARBARA ELIZABETH HOLMES and ELSIE WATCHORN.

From the Biochemical Laboratory, Cambridge.

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The present work is an attempt to approach the study of the metabolism of some growing mammalian tissues. While much work has been carried out on the adult surviving or functioning organ by such methods as perfusion experiments, extirpation of an organ, etc., we know practically nothing of any chemical changes due actually to growth of any particular embryonic organ in the mammal. Probably the only way to study such changes is by means of the technique of tissue culture, where growth takes place *in vitro*. It is through this technique, suitably adapted to our needs, that we have endeavoured to attack the problem. In this communication the changes in ammonia and urea content of the embryo rat kidney are discussed, together with a note on the brain.

Technique.

A technique had to be devised which would give us sufficient material for analytical treatment; it was also necessary to use a liquid culture medium. The routine finally adopted is as follows.

A pregnant doe is anaesthetised and the two horns of the uterus removed under aseptic conditions and placed in two sterile Petri dishes. As much of the work as possible is now done inside a box with a glass top and side doors, similar to that described by Strangeways [1924]. Absolute sterility must be maintained throughout the lengthy processes involved. The embryos are removed from the uterus, decapitated and eviscerated, and, if they are well developed, it is advisable to remove the backbone. The kidneys are removed and placed in a separate dish. The embryonic tissue is chopped as finely as possible, and mixed with Ringer's solution. It is probable that different tissues require Ringer solutions of different composition, but for kidney we have used that described by Pannett and Compton [1924]. The solution is very lightly centrifuged in a hand machine. The upper layer of fluid, which will contain most of the blood cells, is removed, and the minced tissue again mixed with Ringer's solution. It may be necessary again to remove the supernatant liquid layer, but usually hard centrifuging will now leave a faintly opalescent fluid, free from cells. The removal of the blood-cells is most important, as their presence in the culture medium inhibits growth. The cell-free extract thus

obtained nearly always requires further dilution with Ringer's solution, the final strength most suitable for growth being a matter which can only be determined as the result of some experience. A little practice soon enables one to prepare extracts which are remarkably constant in their ammonia content, as the following figures (expressed in mg. ammonia-N per 2 cc. extract) will show:

0·026 0·023 0·026 0·025 0·030 0·022

The kidneys must be freed from all adhering blood, and the number required for each experiment of a series placed in separate dishes, where they are then cut into small pieces. For each experiment we have used 2 cc. of extract, and generally two kidneys. We have taken as much care as possible to use the same amount of tissue throughout any one series of experiments, but owing to the strict aseptic conditions which have to be maintained, it has been found quite impossible to use weighed amounts of tissue. The wet weight of two embryo kidneys from each litter can be determined (*e.g.* 29·5, 44·0, 25·0, 34·0 mg.), and this weight taken as representative of the weight of the remaining kidneys of that litter.

In order that growth may take place, it is necessary for the tissues to have a surface to which they can attach themselves, and for them to be kept moistened and surrounded by the culture medium without being submerged in it. It is thus necessary to use a flask or dish of such a size that the right depth of fluid is obtained when a given quantity of the medium (in this case 2 cc.) is placed in it. In a few of our early experiments we used 100 cc. conical hard-glass flasks with a layer of filter paper on the bottom, but satisfactory growth was not obtained on this, and it was therefore discontinued. Kidney tissue undoubtedly grows best on a glass surface, but if the dish is not absolutely level on the bottom there is danger that a certain amount of tissue will float in the medium, and thus be prevented from growing. We found it almost impossible to obtain dishes or flasks sufficiently even, and on the whole we have found that a very thin layer of cotton-wool has proved quite satisfactory. The pieces of tissue rest on the strands, kept moist by the medium, but in contact with the air. Rubber stoppers or dish covers must be well coated with paraffin wax to prevent evaporation and infection. Incubation at 37° is allowed to proceed for 48 hours. It is generally possible to tell by the naked eye whether or not the culture is sterile after incubation, but as a routine we make smear preparations, which are then stained by Gram's method, and examined with an oil immersion lens. If there should still be any doubt, a drop of the culture medium should be plated out on agar. No analyses have ever been carried out on any but completely sterile cultures.

The culture is filtered into a dry, weighed, hard-glass test-tube, the flask or dish washed with successive small quantities of ammonia-free water, the washings being passed through the filter and added to the filtrate. The weight of the contents of the tube is determined, and aliquot parts are taken for estimation. The filtrate should be kept in the ice-chest till required.

All apparatus for this work must be sterilised, having first been specially cleaned and prepared. In particular, it is important that no trace of alkali should be present on any of the glassware. As a matter of routine we have washed all the glass in sulphuric acid and dichromate, followed by a thorough rinsing in running water, then in distilled water, and finally in ammonia-free water. Each dish, pipette, and cork is separately wrapped in tissue-paper before being sterilised.

For estimation of the ammonia we have used Stanford's method [1923] which we have found to be very accurate until amounts of ammonia-N less than 0·012 mg. are being measured when it is liable to a 10 % error. For urea we have incubated an aliquot part of the filtrate with urease paper [Folin, 1923], and have then estimated the total urea- and ammonia-N, the urea-N being obtained by difference.

Experimental.

An elaborate system of controls is necessary:

A. 2 cc. of medium, without incubation.

B. 2 cc. of medium, plus the same amount of tissue as that used for the growth flasks (*i.e.* generally two kidneys), without incubation.

C. 2 cc. of medium, incubated for 48 hours.

A and B are kept at 0° until required.

Thus the total control will be given by:

$$A + (B - A) + (C - A) = B + (C - A),$$

where B will give the amounts of urea and ammonia nitrogen in the medium and tissue before incubation, and (C − A) will give any due to autolysis of the medium during the 48 hours' incubation. Typical figures obtained for these controls are given in Table I.

Table I.

	Mg. ammonia-N				Mg. urea- + ammonia-N			
Exp.	3	6	9	13	3	9	13	16
A	0·026	0·032	0·026	0·030	0·034	0·050	0·045	0 028
B	0·030	0·041	0·032	0·032	0·033	0·057	0·047	0·038
C	0·032	0·040	0·033	0·038	0·042	0·060	0·044	0·029
Total control	0·036	0·049	0·039	0·040	·0·041	0·067	0·047	0·039

Figures for A and C are for 2 cc. of extract, figures for B are for 2 cc. of extract + two kidneys.

Each experiment consisted of a series of controls and growing preparations, in all of which the same medium and kidneys taken from embryos of the same litter were employed.

It will be seen that there is an increase of ammonia-N in the medium due to autolysis. No increase of urea was found. The amount of urea present in the kidneys is also extremely small, always within the limits of experimental error, so that sometimes no difference at all was obtained between the total urea- and ammonia-N figures and those for ammonia-N alone, occasionally small minus results even were obtained. It is therefore a matter of doubt as to

whether any urea is present or not in the embryonic kidney. The amount present in the embryonic extract was also very small, less than half that of the ammonia-N. The total amount of ammonia- + urea-N as estimated is of course outside the limits of experimental error, but the difference due to urea-N alone falls within that limit.

In addition to these controls, we devised a further control (D), which we have called the "resting tissue." This consists of the same amount of extract and tissue as the contents of the growth flasks, and like them is incubated for 48 hours. But by using a smaller flask for this resting tissue, we have prevented it from attaching itself to the surface of the glass, and thus also prevented growth from taking place. We have aimed at such a depth of fluid that, although the tissue is freely movable in it, it is only just submerged, and is able to obtain sufficient oxygen to prevent its death. It is obvious from the results of this resting tissue, compared with the total control (Table II), that autolysis has not taken place. We therefore concluded that the tissue was still living, as autolysates of adult mammalian kidneys form both ammonia and urea [McCance, 1924; Luck, 1924, 1]. It was, however, important to make sure of this point; so on one or two occasions at the end of 24 hours' incubation, the flask containing the resting tissue was opened, and part of the medium with some of the pieces of floating tissue was removed to a larger flask, and again sealed. The few pieces remaining behind in the original flask were stained and examined. No sign of growth could be detected. The new flask now contained such an amount of fluid that the remaining pieces of tissue could, if they were still living, attach themselves to the surface of the glass. After a further 24 hours' incubation they were stained and examined, and gave unmistakable evidence of growth, budding having taken place quite freely. This resting tissue having been proved to be living but non-growing tissue serves as a valuable control to the flasks in which growth has taken place. Any difference between ammonia- and urea-N in the growing and resting tissue, both having been subjected to the same conditions and compared with the "total control," can with some confidence be attributed to the actual growth of the tissue. The metabolic activity of the resting tissue seems to be reduced to an absolute minimum consistent with life, and may be described as its resting metabolism.

Table II. *Resting tissue compared with total control.*

	Resting tissue mg. ammonia-N + urea-N	Total control mg. ammonia-N + urea-N
Exp. 13 a	0·063	0·055
13 b	0·050	0·055
13 c	0·039	0·040
19	0·064	0·062

In view of the fact that the resting tissues must be considered to be living for at least the first 24 hours of the experiment we were interested to find a marked difference between these and the growing tissues as regards their activity in producing ammonia and urea. This has already been stated in a

preliminary paper [Holmes and Watchorn, 1927] and can therefore be discussed only very briefly here. In the case of the floating tissue the amounts of ammonia and urea after two days' incubation are exactly the same as the amounts contained in the total control. Examples are given in Table II, to which attention has already been drawn, and they show that the tissue has produced no ammonia or urea.

The growing tissue, on the contrary, produces considerable amounts of ammonia or urea or both together, as may be seen from the figures given in Table III. One might expect that, if this occurred to any extent during the life of the embryo, some sign of it would be found even in the floating tissues, but it must be remembered that the tissue has been allowed to become cold in the interval necessary for the preparation of the medium and the planting of the tissues. Cooling is very effectual in causing cessation of cell division in mammalian cells, and thus, if this production of ammonia and urea is associated with growth, as we believe it to be, it would only be found in cases where the growth had begun again.

In order to compare the results given by the resting and by the growing tissues, it was necessary to be certain that these latter had, in fact, grown. It was not, of course, possible to stain and examine preparations, and afterwards use them for estimation. We have sometimes resorted to the plan of growing special controls for staining, and we have sometimes been able to stain fragments of tissue which remained sticking to the glass after the vessel had been washed out several times for estimation purposes. As a general rule it is quite possible to detect growth with the aid of a binocular dissecting microscope without staining (the vessel merely being placed on black paper), and by this means it is possible to examine preparations in which the ammonia and urea are later to be estimated.

As it happens, in the case of embryo kidney tissue it has been found so consistently that tissues which are not growing form no urea or ammonia, that when these substances increase in amount, it may almost be assumed that growth has occurred. Floating preparations in which a few fragments of tissue have stuck to the glass and grown, show small increases in ammonia and urea. On the other hand, if a "growing" preparation fails to grow (this happened on two occasions, and the failure was eventually recognised as being due to an insufficiency of magnesium in the Ringer's solution), no increase in either of these substances is found.

The results of our experiments with "resting" kidney tissue agree well with those obtained by Warburg [1924] with sliced adult rat kidney tissue. He found that a considerable amount of ammonia appeared as the experiment progressed; but finding that this apparent production of ammonia by the kidney tissue had a very small temperature coefficient, he concluded that he was not dealing with a chemical process, but that the ammonia was merely being washed out of the tissue. This ammonia, which he considered to be existing preformed in the tissue, would be estimated in our tissue control B.

Table III.

Resting or control		Growing tissue		Increase	
Ammonia-N (mg.)	Urea-N (mg.)	Ammonia-N (mg.)	Urea-N (mg.)	Ammonia-N (mg.)	Urea-N (mg.)
0·036	0·011	0·075	0·034	0·039	0·023
0·036	0·011	0·058	0·012	0·022	Nil
0·039	0·029	0·070	0·023	0·031	Nil
0·055	0·022	0·096	0·039	0·041	0·017
0·055	0·022	0·060	0·055	Nil	0·033
0·058	0·015	*0·050	0·033	*Nil	0·018

* Poor growth.

It will be seen from the examples given in Table III that the amounts of ammonia and urea produced during growth are considerable, and far beyond the limits of the experimental error of this method. It is quite usual for the extra amount of ammonia produced to be equal to 0·1 % or more of the original wet weight. As examples the following experiments may be quoted:

Exp. 18 *c*. Wet weight 34 mg. Urea increased 0·033 mg.
Exp. 3 *a*. Wet weight 33 mg. Urea increased 0·023 mg.
Ammonia increased 0·039 mg.

We have no data as yet which might be helpful in showing to what chemical processes we must relate the production of these metabolites. Presumably proteins or similar substances (Carrel and Baker [1926] claim that, in the case of chick tissues growing *in vitro*, proteoses are the most important nutritive constituent of the medium) contained in the embryo extract must be broken down to their constituent amino-acids to be rebuilt into the cells of the growing tissue. Deamination of the amino-acids not required for the synthesis of cell proteins might then occur. If the deamination represents protein combustion for the purpose of obtaining energy for growth, we should be able to prevent this to a considerable extent by adding glucose to the medium, and this we propose to attempt shortly.

Nash and Benedict [1921] showed that during life the kidneys were capable of producing very considerable amounts of ammonia when the glomerular filtrate was acid. The mechanism of this ammonia production is not known. It was attempted at one time to account for it by assuming the presence of urease in the kidney, but no urease could be found when experiments were carried out *in vitro*. In our work we find no evidence for the existence of kidney urease, since the relative and absolute amounts of urea and ammonia present remain unchanged during 48 hours' incubation of our "resting" tissues.

There is, of course, no reason for assuming that the mechanism of this ammonia production is the same as the mechanism of its production by growing tissues. In fact in our growing tissues it seems that it may be urea or ammonia which is produced, or even both together (see Table III), and, according to the evidence of Bollman, Mann and Magath [1924] adult functioning kidney produces no urea. It should be pointed out that in the experiments of Bollman, Mann and Magath the kidneys were plentifully supplied

with glucose, which would tend to prevent urea formation if this originated in protein breakdown. We may further find that a supply of glucose to our growing tissues prevents the formation of urea in them also, or it may be that the metabolism of the growing tissue is in this respect quite distinct from that of the functioning adult tissue.

Experiments with brain tissue.

In addition to the experiments just described on kidney tissue, we have carried out the same technique using embryo brain tissue, and while we have not progressed far enough in this part of the work to allow any very definite conclusions to be drawn, one or two points seem to be quite well established and may be worth describing here, though later we hope to be able to deal with brain tissue in more detail.

In the case of brain tissue we have found it more difficult to obtain good growth, and to be certain when growth has occurred. In the experiments with kidney tissue we found it expedient, owing to the small size of the kidneys, to use fairly old embryos, and these gave very good growth. In the case of brain tissue, however, we found it more satisfactory to use young embryos, and even in these one cerebral hemisphere provides plenty of tissue.

The "resting" preparations gave results similar to those obtained with "resting" kidney tissue, in that the ammonia- and urea-N, taken together, showed no increase over the calculated amount in the total control, in fact, slight decreases were observed.

One remarkable fact was noticed, namely, that there was always some suggestion of an increase of ammonia at the expense of urea, that is, a suggestion of urease action. To obtain definite evidence of this, it is necessary to keep the filtrate from the ice-chest tissue control as cold as possible until it is required for estimations. Otherwise the urea in this control will also decrease in amount.

When such precautions are taken, results similar to those given in Table IV are obtained.

Table IV.

	Resting tissue (mg.)	Total control (mg.)
Ammonia-N	0·053	0·033
Urea-N	0·015	0·038
Ammonia-N + urea-N	0·068	0·071

So far the occurrence of urease in the tissues of higher animals has only been reported for the gastric mucosa of certain species [Luck, 1924, 2], but we have no reason so far to doubt that it is actually present in rat brain tissue, although probably only in small amounts. Adult rat brain tissue has been found to convert a little added urea to ammonia, but only a small number of experiments have been carried out. Rabbit brain tissue gave definitely negative results, showing no sign at all of the presence of urease. We have injected several rats subcutaneously and intraperitoneally with urea. With 1 g.

of urea the rats showed rigidity of the tail, twitchings and finally drowsiness, but with more urea (about 2 g. given at intervals) severe convulsions occurred about three-quarters of an hour later. So far as we know this effect has not been described for other animals known to contain no urease, as for example the rabbit; and while we cannot yet state that the effect we obtained in the case of the rat was due to the conversion of urea into ammonia, the possibility must be considered and further investigated.

In the case of the growing preparations we have obtained only a few satisfactory results, and these are most unlike those obtained with growing kidney tissue. When good growth has taken place we have usually found a considerable fall in the ammonia- and urea-N taken together. This suggests that some synthesis of nitrogenous substances at the expense of ammonia and urea must take place when the tissue is actively growing. More experimental data will have to be obtained, however, before this view can be expressed with certainty, and this work is now in progress.

Summary.

1. The technique of tissue culture has been employed to study the nitrogenous metabolism of growing tissues.

2. When growing, embryonic rat kidney tissue produces considerable amounts of ammonia and urea.

3. "Resting" embryonic kidney tissue, incubated under exactly the same conditions as the growing tissue, produces neither ammonia nor urea

4. Some preliminary experiments with brain tissue are described and the results contrasted with those obtained with kidney tissue. The probable existence of urease in the rat brain is discussed.

In conclusion we wish to express our indebtedness to Mr R. A. McCance, who was working with one of us (B. E. H.) for more than a year at this investigation when it was first undertaken, and to whom we owe many points of technique and many suggestions.

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