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

II. EFFECT OF GLUCOSE UPON THE AMMONIA AND UREA PRODUCTION OF KIDNEY TISSUE.

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In the first paper of this series [Holmes and Watchorn, 1927] we described the formation of ammonia and urea by the tissue of embryonic rat kidneys when this was growing *in vitro*. The formation of these substances occurred only when actual growth took place, and we were inclined to believe that it indicated the combustion of nitrogenous substances to supply the energy necessary for growth. If this were the case, the addition of glucose, *i.e.* the supplying of another and probably more available source of energy, might be expected to lessen the production of ammonia and urea during the growth of the tissue.

These experiments were undertaken to discover whether such an effect is produced by adding glucose to the culture medium.

Technique.

This has been essentially the same as that previously described [Holmes and Watchorn, 1927]. Some modifications of Stanford's ammonia method [1923] have been introduced. We have used much hotter water for surrounding the distilling flask—namely about 60°. If the apparatus has just been well evacuated, this enables brisk distillation to proceed for 12 minutes, or often longer, without the apparatus being attached to the filter pump. It should, however, be connected to the pump for two or three minutes before finally discontinuing distillation, otherwise a slightly low result is apt to be obtained. When letting down the vacuum, air should be admitted through a sulphuric acid wash-bottle. At no period during distillation do we pass air through the apparatus. If cooler water is used, as described by Stanford, attachment to the filter pump is necessary after about 3 minutes, and under these conditions we have found that usually only 90 % of the ammonia distils over. We have assured ourselves that, at any rate for our solutions, the hotter water is no disadvantage.

Glucose determinations have been made by Hagedorn and Jensen's [1923] method. Hydrogen ion concentration has been measured by aid of the B.D.H. capillator, using bromothymol blue as indicator, which completely covers our range.

Experimental.

Pannett and Compton's [1924] Ringer solution was used for diluting the embryo extract. Varying amounts of glucose have been added at different times. As we previously showed that no ammonia or urea was formed by resting tissue (*i.e.* tissue which was living, but was floating freely in the medium and thus unable to grow) after 48 hours' incubation at 37° we have frequently discarded the elaborate system of controls formerly used, and have simply taken the resting tissue preparations as controls for the growing tissues. Preliminary experiments with resting tissues have shown that when kidneys from the same litter are used, the addition of glucose to an otherwise similar medium does not affect the non-occurrence of ammonia or urea formation by non-growing, non-autolysing tissue (Table I).

Table I.

	Mg NH_3-N		Mg urea- + NH_3-N	
Exp.	Without glucose	*With glucose	Without glucose	*With glucose
20	0·036	0·034	0·060	0·061
22	0·032	0·032	0·062	0·061
29	0·048	(i) 0·046 (ii) 0·042	0·084	(i) 0·087 (ii) 0·083
30	—	—	0·056	(i) 0·053 (ii) 0·055
32	0·044	0·046	0·114	0·119
34	0·027	0·026	0·082	0·075

* The original concentration of glucose in these cases is from 0·07 to 0·09 %.

Having established this fact, it was then possible to use resting tissue preparations containing glucose as controls for growing tissue.

The addition of glucose gave rise to a marked alteration in the metabolism of the growing tissue, in that it caused a very definite inhibition of ammonia and urea formation. Unfortunately, as the number of embryos in a litter is sometimes small, it has not always been possible to include preparations of growing tissues in plain and in glucose media in the same series. This is not a very serious drawback, as we have previously obtained a large number of experimental results showing that ammonia and urea are formed when the tissue grows in the absence of glucose. In the presence of glucose the formation of these substances is, as is shown by comparison with the resting controls, frequently completely inhibited. Under these circumstances, when the results obtained are exactly similar in growing preparations and in controls, it is necessary to be certain that growth has actually taken place. Even if tissue without glucose is included in the same series, and is known to have grown well, there is obviously no certainty that the glucose-fed tissue has also grown, as the presence of the sugar may either inhibit or accelerate growth. Except in the early experiments, we have therefore included in each series one or more small control dishes containing small amounts of tissue growing in the same medium, which could be used for staining and microscopical

examination. These revealed the fact that very small amounts of glucose in the medium did not inhibit, but if anything aided growth. In some cases better differentiation seemed to take place in the presence of glucose than in its absence. However, on one or two occasions when a concentration of over 0·2 % was present, inhibition of growth and even death of the tissue apparently took place[1]. This concentration is, of course, remarkably low compared with figures given for possible glucose concentrations by other observers [Lewis, 1922; Willmer, 1927], who find 1 % and even 2 % solutions suitable for growth. This difference may be due to the fact that we have used mammalian tissue, whereas other observers have dealt with chick tissue. The amount of glucose in the egg is fairly large at some stages of its development, and it is possible that the tissues of the embryo are exposed to relatively high concentrations. Indeed, the blood-sugar in embryo chicken blood is about 0·2–0·3 % at some stages of development [Hanan, 1925]. Willmer [1927] states that 0·2 % would be the best strength for the *in vitro* growth of chick intestine if it could be constantly maintained. In the case of embryo rat kidney we find that concentrations approaching mammalian blood-sugar level (*i.e.* 0·07 to 0·11 %) are more suitable.

Figures for tissues growing in a glucose medium are given in Table II. The concentration of glucose originally present was from 0·07 to 0·11 %.

Table II.

	Control		Growing tissue	
Exp.	NH_3-N	NH_3- + Urea-N	NH_3-N	NH_3- + Urea-N
20	0·036	0·060	0·039	0·064
22	0·032	0·062	0·033	0·052
			0·024	0·051
29	0·048	0·084	0·042	0·069
			0·039	0·069
30	0·028	0·066	0·028	0·067
33	0·046	0·120	0·046	0·108
34	0·026	0·075	0·027	0·055
			0·029	0·072
35	0·028	0·055	0·026	0·057
36	—	0·079	—	0·080

It will be seen that, in some experiments, the growing tissue has the same figure for ammonia and urea as its control, but that in others (Exps. 22, 29, 33 and 34) the total ammonia- and urea-nitrogen is actually less in the growing preparation than in the resting tissue. It is perhaps significant that these were all flasks in which good growth had taken place. When tissues grown in plain and in glucose media are included in the same series (that is to say, when all kidneys used are taken from the same litter, and the media made from the same embryo extract) then the results are even more striking (Table III).

[1] The glucose solutions used in these cases had been autoclaved, and their toxicity may be due to this.

Table III.

Exp.	Resting control		Tissue growing in absence of glucose		Tissue growing in presence of glucose		Increase in NH_3- + Urea-N	
	NH_3-N	NH_3- + Urea-N	NH_3-N	NH_3- + Urea-N	NH_3-N	NH_3- + Urea-N	Without glucose	With glucose
20	0·036	0·060	0·050	0·079	0·039	0·064	0·019	Trace
			0·075	0·075			0·015	
28	0·045	0·076	0·060	0·105	—	0·092	0·029	0·016
30	0·028	0·066	0·050	0·115	0·028	0·067	0·049	Nil
35	0·028	0·055	0·027	0·084	0·026	0·057	0·029	Nil
				0·085			0·030	

The concentrations of glucose originally present vary from 0·07 to 0·11 %.

In Exp. 30, for instance, which is shown in this table, the growth in the glucose medium was particularly good, yet the content of ammonia- and urea-nitrogen showed no rise, whereas in the plain medium the growth of the tissue caused a 74 % increase in the amount of ammonia and urea present. Exp. 35 in Table III gives another very definite illustration of the effect of the addition of glucose.

We had hoped when this work was first begun that we might find a striking quantitative difference in the carbohydrate metabolism of the floating (resting) and the growing tissues. Up to the present we have only attempted to estimate the disappearance of glucose, and the results have been somewhat disappointing, as there is often no such difference found. As might have been expected from the work of Warburg and others, the resting kidney tissue itself is active in breaking down glucose; thus, anything which delays the commencement of growth or diminishes its extent must tend to obscure any quantitative differences between the resting and the growing preparations. Again, estimations showing the disappearance of glucose from the medium give no indication of the manner in which the sugar may be utilised. It is possible, for instance, that the breakdown of the glucose may be far more complete, and the energy obtained therefore far greater in amount, in some preparations than in others. It may even happen that further breakdown of glucose occurs after the flasks are removed from the incubator, and we have taken no special precautions to prevent this.

Although in many cases the resting tissue has caused as much glucose to disappear as the growing tissue, it has been possible in experiments where very good growth has occurred to demonstrate an extra glucose breakdown on the part of the growing tissue. Thus, in Exp. 30, the original amount of glucose present in each flask was 1·5 mg., the amount remaining in the control preparation 0·9 mg., and the amount remaining in the growing preparation only 0·7 and 0·4 mg. The glucose estimations are carried out on small portions of the filtrates in which the ammonia and urea values are determined. A control flask is kept at − 2° for the duration of the experiment and is used for estimation of the amount of glucose originally present; we

have usually found that about one-third of this amount is broken down by the resting tissue.

p_H determinations have also been carried out on the filtrates. These have been found to give a rough check on the figures we obtain for glucose breakdown, and are more generally useful in determining the optimum hydrogen ion concentration for tissues growing under our particular conditions.

Discussion.

We had previously shown that kidney tissue which was growing in a plain embryo extract always formed considerable amounts of urea and ammonia, though resting (floating) tissue under identical conditions did not.

We have now found that, in the presence of glucose, very good growth may occur without any production of urea and ammonia by the growing tissue.

It seems probable, therefore, that, in the absence of glucose, nitrogenous substances are broken down to provide the energy necessary for the growth of the tissue, thus giving rise to the ammonia and urea which appear. When glucose is present the necessary energy may be obtained instead from the breakdown of glucose.

In a great many cases we have obtained very good growth in glucose media without any increase (sometimes even a slight decrease) in the ammonia- and urea-nitrogen. Sometimes a slight but definitely lessened production of these substances is found, while on one occasion the increase in ammonia- and urea-nitrogen seemed to be unaffected by the presence of the glucose, although it is probable that in this case the extent of the growth was greater in the glucose medium than in the plain.

We have no explanation for these variations, which may be due to differences in the age of the embryos, alterations in the glucose during sterilising, or varying proportions in the media of glucose and the nitrogenous substances which are broken down by the tissue. There is no doubt, however, that we have obtained very definite evidence of the so-called "protein-sparing" action of glucose.

Raistrick [1921], dealing with the effect of glycerol upon the breakdown of tryptophan by bacteria, claims that glycerol cannot be said to "spare" the amino-acid, since, although less ammonia is formed when the glycerol is present, a larger amount of the nitrogen from the tryptophan is built up into the bacterial tissues. Raistrick considers that the effect of the addition of glycerol is to produce a more suitable carbon/nitrogen ratio for bacterial growth. Even in this case, however, there was (this was pointed out to us by Miss M. Stephenson) more tryptophan left untouched at the end of the experiment when the bacteria were grown in the glycerol medium, than when they were grown in a medium containing tryptophan alone, so that some actual sparing of the amino-acid did occur. We are inclined to believe that in our case the glucose has much this effect, since, although the oxidative

breakdown of nitrogenous substances (or that part of it which is manifested by the appearance of ammonia and urea) is largely prevented, the growth is probably greater in extent, and therefore more nitrogen must be removed from the medium for purposes of synthesis.

Lewis [1922], Willmer [1927] and others have employed glucose in culture media, and consider it useful in maintaining growth. Lewis, in fact, finds that it much prolongs the life and activity of cells which are allowed to grow for some time in the same drop of culture medium. It will be seen from consideration of the facts set forth in this paper that this prolongation of life and the power of growth may be due to the addition of extra carbohydrate fuel, or to the prevention of the accumulation of ammonia and possibly other products of protein breakdown. It is highly probable that unduly high concentrations of ammonia are very toxic for tissue cells. This is in fact indicated by the work of Krontovski and Radzimovska [1922] who showed that exposure of tissues for half an hour to Michaelis' ammonia standards inhibited the subsequent growth of the cells far more than the alkalinity of the solutions warranted.

In a previous paper [Holmes and Watchorn, 1927] we discussed the apparent contradiction between our results and those of Bollman, Mann and Magath [1924] who found that the liver is the only organ in the body which produces urea. The growing embryonic kidney can, however, produce ammonia, urea or both together. On the other hand, the presence of a little glucose frequently prevents the appearance of these substances, and in the experiments of Bollman, Mann and Magath the blood-sugar was kept at a normal, even slightly supernormal, level. Thus it is not possible to state at present that the formation of urea occurs only when the kidney tissue is growing, and not in the adult, functioning kidney. The origin of the ammonia, which is excreted by functioning kidney when the glomerular filtrate is acid, is not known.

What has been commonly spoken of as the "protein-sparing" power of carbohydrates is in the intact animal a complex phenomenon. It seems of interest to have demonstrated directly such an action in the case of an isolated tissue removed from the immediate influence of insulin and other hormones which affect the metabolism in the body of the whole animal.

Summary.

(1) Addition of glucose to the medium makes no difference to the non-occurrence of ammonia and urea production by non-growing, but surviving embryonic rat-kidney tissue.

(2) Addition of glucose to embryonic rat-kidney tissue growing *in vitro* generally inhibits the production of ammonia and urea which would otherwise take place, and frequently actually causes a reduction in the amount of these substances initially present.

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