The Effect of Adrenal Cortical Preparations Added *in vitro* upon the Carbohydrate Metabolism of Liver Slices

1. THE EFFECT OF ADRENAL CORTICAL EXTRACT (ESCHATIN) UPON SYNTHESIS OF GLYCOGEN AND OF TOTAL CARBOHYDRATE

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(*Received* 15 *August* 1949)

It was shown by Seckel (1940) that addition of adrenal cortical extract ('eschatin', free from preservative) to buffered physiological salt solution in which liver slices were incubated caused decrease in glycogen disappearance. The incubation was carried out in air, and the 'percentage inhibition of glycogenolysis' at the end of 1 hr. was 21–67. This well-marked effect of the extract *in vitro* seemed worthy of further investigation, especially with the aim of getting some light on the enzyme mechanisms concerned in it. We have confirmed Seckel's results (Chiu & Needham, 1948), and extended the experiments to include other conditions of incubation, the estimation of total carbohydrate as well as glycogen, and the use of certain pure adrenal cortical steroids. The work with the latter is described in the second paper of this series (Chiu, 1950). Experiments carried out in an atmosphere of nitrogen showed that the inhibitory effect of the eschatin was entirely absent then. It therefore appeared that the effect of the extract was in favouring synthesis of glycogen, the observed degree of glycogen disappearance in air being the resultant of simultaneous breakdown and synthesis. This view of the liver slice as the seat of both glycogen breakdown and formation has been expressed by previous workers, e.g. Bendall & Lehmann (1941) in considering the effects of adrenaline *in vitro*. Deane, Nesbitt, Buchanan & Hastings (1947) have indeed shown histologically that, with rat- and rabbit-liver slices 0·5 mm. thick, incubated in medium containing glucose or pyruvate under an atmosphere of 95% oxygen–5% carbon dioxide, glycogen had disappeared from the cells of the interior, while new formation had gone on at the periphery of the slice. This picture was found even when estimations showed that an overall loss of glycogen, compared with the initial value, had taken place.

In the next series of experiments we used conditions favourable to the demonstration of glycogen synthesis—presence of substrates such as pyruvate, lactate or glutamate and an atmosphere of oxygen; when increase of glycogen or of total carbohydrate content was observed in the control, the amount of increase was always greater in samples containing eschatin.

EXPERIMENTAL

Preparation of tissue. The rats used were piebald Norwegian, all males, weighing 150–300 g. Both rats and rabbits were anaesthetized with nembutal (for rats, 9 mg./100 g. intraperitoneal; for rabbits, 13 mg./kg. intravenous); the liver was removed and slices of thickness 0·3–0·4 mm. were prepared, always from the same lobe, either free-hand or using the slice-cutter of Stadie & Riggs (1944). The slices were not immersed in any salt solution, but were placed when cut in a covered glass chamber arranged over ice. In order to ensure uniform sampling the slices as cut were placed serially in a number of piles equal to the number of samples needed; 100–150 mg., made up of about four slices, were used for each sample. When the slice-cutter was used and large slices were obtained, these were cut into portions and treated in the same way as the smaller slices. (Less satisfactory duplicates were obtained if a single large slice was used for each sample.) The samples were weighed at the beginning of the experiment on a torsion balance. Samples in duplicate were used throughout. The average difference between duplicates was as follows: for glycogen estimations ±5·8%, extreme range ±20% or (when the slice-cutter was used) ±4% and extreme range ±11%; for total carbohydrate estimations by method (*a*) below, ±5%, extreme range ±15%; for total carbohydrate by method (*b*) below, ±2·7%, extreme range ±9%.

Conditions of incubation. Three different salt solutions were used. (*a*) Seckel's phosphate-Ringer (1940); (*b*) Krebs's bicarbonate-Ringer (Krebs & Henseleit, 1932); (*c*) Ringer solution containing 0·006 M-phosphate. Solution (*a*) was used for the experiments on glycogenolysis; (*b*), which contains only 0·0012 M-phosphate compared to 0·0066 M in (*a*), was used in experiments aimed at showing glycogen synthesis, since decreasing free phosphate concentration promotes removal of phosphate from glucose-1-phosphate and formation of glycogen; (*c*) was used in experiments where O_2 uptake was followed, since it contains no bicarbonate. In all cases the initial pH was 7·4, an atmosphere of 95% O_2–5% CO_2 being used with (*b*), air, N_2 or O_2 with (*a*) and (*c*).

The slices (100–150 mg.) and medium were placed in Barcroft or Warburg flasks attached to manometers and shaken continuously at 37°. At the end of the experiment, when glycogen was to be estimated, the slices were transferred as rapidly as possible to 26·6% (w/v) KOH. When total carbohydrate was to be estimated, H_2SO_4 was added to the entire contents of the flasks to make the concentration N. When glucose was to be estimated in the fluid,

samples of known volume were removed; these were immediately either precipitated with $ZnSO_4$ and $Ba(OH)_2$ for Nelson's (1944) method, or brought to pH 5·8 in preparation for the glucose oxidase method and stored temporarily at 0°. The slices for initial values of glycogen or total carbohydrate were placed in 26·6% (w/v) KOH or N-H_2SO_4 at the time the experimental samples went into the bath.

Active extract. The preparation used was eschatin, a purified extract of adrenal cortex made by the method of Swingle & Pfiffner (1931). Samples free from preservative, 1 ml. equivalent to 90 g. fresh adrenals, were kindly supplied by Parke, Davis and Co. The amount used was 0·15 ml./ml. medium; this corresponds approximately to the amount used by Seckel (1940), who added twice this volume of an extract, 1 ml. of which was equivalent to 40 g. fresh adrenal. Like Seckel, we found this dose gave maximal effect; when the dose was reduced to one-third, no inhibitory effect was obtained.

Chemical methods. Glycogen was separated by the method of Good, Kramer & Somogyi (1933) and the glucose formed on hydrolysis was estimated by the method of Miller & Van Slyke (1936). In the beginning of the work attempts were made to use the colorimetric method of Van Wagtendonk, Simmons & Hackett (1946). It was found, however, that with liver of low glycogen content negative results were obtained, i.e. readings lower than those of the reagent blank. It thus appears that the glycogen prepared according to this procedure contains (at any rate in some circumstances) material which can vitiate the results, probably by reacting with the iodine.

'Total carbohydrate' was estimated in three different ways. (*a*) The whole contents of the flask (slices + medium) were hydrolysed in N-H_2SO_4 for 2·5 hr. at 100°. After neutralization, precipitation with $ZnSO_4$ and $Ba(OH)_2$ was carried out and the reducing substance in the filtrate was estimated according to Nelson (1944), using a Gallenkamp photoelectric colorimeter. (*b*) After acid hydrolysis as above and adjustment to pH 5·8, the glucose was estimated by means of glucose oxidase, the O_2 uptake being measured in Warburg manometers under the conditions specified by Keilin & Hartree (1948). (*c*) Glycogen was estimated in the slices and glucose in the medium by Nelson's method or the glucose oxidase method. If supplies of glucose oxidase are available, method (*b*) is to be preferred; with method (*a*) it was often difficult to obtain final solutions entirely free from opalescence, since traces of fatty material passed into the $ZnSO_4$–$Ba(OH)_2$ filtrate. Method (*c*) does not necessarily give the whole of the carbohydrate present, but it is useful in that the two fractions which probably make up much the greater part can be estimated with accuracy.

Adenosinetriphosphate was estimated by making a trichloroacetic acid extract at 0° and precipitating with Ba. Nine-minute P was determined in the precipitate, after removal of Ba, by Fiske & Subbarow's method (1925).

RESULTS

The results with liver slices (from well-fed rats) incubated with an atmosphere of air are shown in Table 1. Two experiments with rabbit liver are included.

In Table 2 are shown the results when an atmosphere of nitrogen is substituted. The 'inhibition of glycogenolysis' seen in Table 1 in presence of the extract is absent here, indeed, there is usually rather more loss of glycogen in the samples containing the extract, probably on account of the presence of traces of organic solvent in the eschatin. In order to obtain complete abolition of the inhibition, it is essential that the air in the flasks should be completely displaced by nitrogen purified by passage over heated copper. This is not surprising in view of the observation of Dixon & Elliott (1929) that the oxygen uptake of liver slices is as great at 7% partial pressure of oxygen as at 100%.

Table 1. *The effect of eschatin on glycogen content of liver slices, incubated aerobically*

(About 100 mg. of slices were suspended in 2 ml. of Seckel's Ringer under atmosphere of air and incubated 60 min. at 37° with shaking. Results expressed as 'percentage inhibition of glycogenolysis'.)

Date	Glycogen content (mg./100 mg. wet tissue)			Percentage 'inhibition'
	Initial	Final control	Final + eschatin	
		Rats		
27 July 1946	1·60	0·57	1·29	69
29 July 1946	4·60	0·71	1·48	20
25 Oct. 1946	1·66	0·48	0·89	35
27 Aug. 1947	4·27	0·86	2·61	51
7 Oct. 1947	6·34	1·02	2·15	21
11 Oct. 1947	2·67	0·73	0·94	11
29 Oct. 1947	0·94	0·14	0·19	6
12 Feb. 1948	1·31	0·31	0·46	15
22 Aug. 1948	0·86	0·62	0·69	28
6 Mar. 1949	2·29	0·34	0·46	6
8 Mar. 1949	5·84	2·14	2·67	15
10 Mar. 1949	2·57	0·49	0·71	11
		Rabbits		
9 Oct. 1947	1·36	0·74	0·80	9
1 Mar. 1947	2·07	0·98	1·56	53

Table 2. *The effect of eschatin on glycogen content of rat liver slices, incubated anaerobically*

(About 100 mg. of slices were suspended in 2 ml. of Seckel's Ringer under atmosphere of N_2 and incubated 60 min. at 37° with shaking. Results expressed as 'percentage inhibition of glycogenolysis'.)

Date	Glycogen content (mg./100 mg. wet tissue) Initial	Final control	Final + eschatin	Percentage 'inhibition'
15 Aug. 1946	2·80	0·33	0·14	−8·7
19 Aug. 1946	5·74	0·96	0·66	−6
21 Aug. 1946	3·12	0·94	0·72	−9
21 Oct. 1947	1·27	0·38	0·38	0
8 Mar. 1949	5·84	1·42	1·44	+1
10 Mar. 1949	2·57	0·87	0·82	−2

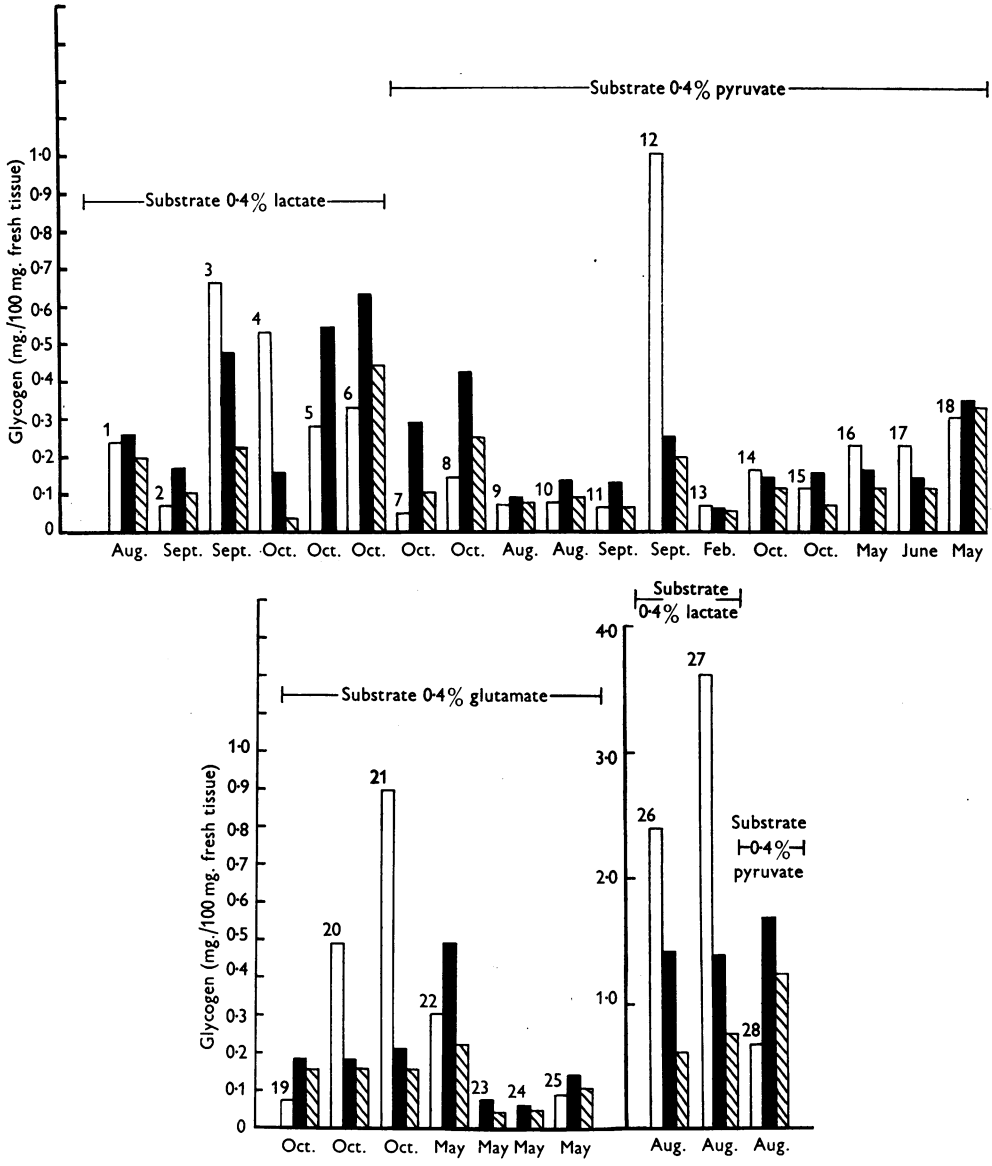

Fig. 1. Glycogen changes in rat-liver slices with and without added eschatin. Animals fasted 24 hr. Incubation, 1 hr. at 37°. Medium, Seckel's Ringer for nos. 1–8, 26 and 27. Krebs's Ringer for nos. 9–25 and 28. Gas phase: O_2 or 95 % O_2–5 % CO_2 (according to medium). Unshaded = initial value; black = value after incubation with substrate + eschatin; shaded = value after incubation with substrate only.

For the experiments summarized in Figs. 1–3, the usual conditions for demonstrating carbohydrate synthesis were used—the slices were prepared from the livers of fasted animals, substrate was added to the medium and the gas phase was pure oxygen or 95 % oxygen–5 % carbon dioxide. All the experiments done are included, and it will be seen that in every case the glycogen or total carbohydrate content is greater in the presence of the active extract.

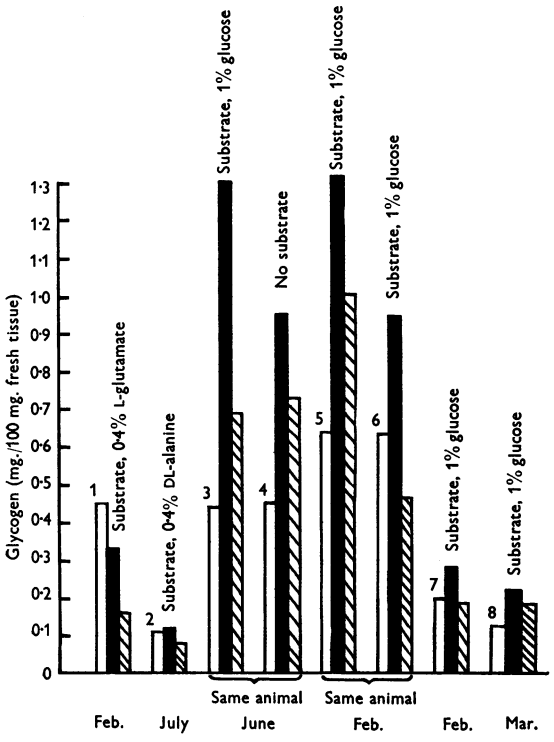

Fig. 2. Glycogen changes in rabbit-liver slices with and without eschatin. Animals fasted 48 hr. Incubation, 1 hr. at 37°. Medium: Krebs's Ringer for nos. 1 and 3–5; phosphate-Ringer for nos. 2 and 6; bicarbonate-Ringer for nos. 7 and 8. Gas phase: O_2 or 95 % O_2–5 % CO_2 (according to medium). Unshaded = initial value; black = value after incubation with substrate + eschatin; shaded = value after incubation with substrate only.

In all this work great individual variation was observed both as regards the initial carbohydrate content of livers from rats treated in the same way (fasted the same length of time or fed on the same diet) and as regards the ability of the liver slices to synthesize glycogen. The same variability is seen in the degree of inhibition of glycogenolysis which again probably depends on differences in power to synthesize carbohydrate. It is noticeable that the effect of the hormone on carbohydrate synthesis as judged by inhibition of glycogenolysis is often greater than any effect observed in the experiments designed to demonstrate synthesis. This may be connected with the fact that livers of well-fed animals were used for the glycogenolysis experiments, while livers of fasted animals were used for the experiments of Figs. 1–3. When the initial glycogen of the fasted liver fell very low (below about 0·1 %) little synthesis in the controls and little effect of eschatin was seen.

It has been noted by Verzár & Wenner (1948*b*) that minced muscle or liver, prepared from starved rats and containing very little glycogen, forms no glycogen from added glucose-1-phosphate unless glycogen is added too. Stadie & Zapp (1947) observed that diaphragm with high initial glycogen content from well-fed rats has a much greater capacity for forming glycogen from glucose than has comparatively glycogen-poor diaphragm from fasted rats. What part is played in these phenomena by such factors as loss of enzymes or coenzymes during inanition, or lack of glycogen deposits to act as nuclei for fresh glycogen formation (Cori, Swanson & Cori, 1945) remains to be investigated.

We did not observe in these experiments, extending over nearly 3 years, any correlation between the time of year and the synthetic power of the liver slices or the effect of the adrenal cortical preparations on the synthetic power. Seasonal variations in metabolic activities of this sort are, however, not uncommon: Lehmann & Bendall (1949), for example, found with the rabbit much greater power of glycogen synthesis in liver slices during the summer than during the winter. We have, therefore, noted the month against each experiment, in case such data might prove of interest in some larger survey of seasonal effects.

DISCUSSION

Observations on adrenalectomized animals have shown that exposure to stress leads readily to hypoglycaemia and depletion of glycogen stores in liver and muscle. Further, injection of cortical extract into fasted animals leads to an increase in the carbohydrate stores accompanied by an increase in nitrogen excretion always great enough to allow of the hypothesis that the new carbohydrate has been formed at the expense of protein. The view has, therefore, often been discussed that certain adrenal cortical steroids are concerned with formation of carbohydrate from tissue protein (see Long, 1942; Hartmann, 1942). As Long has pointed out, the steroids might intervene (*a*) in mobilization of amino-acids from tissue protein; (*b*) in deamination of amino-acids; (*c*) in formation of glucose from the residues after deamination. He suggests also a further possibility based on the results of Seckel (1940): that inhibition of glycogen breakdown by excess of steroid might lead to immobilization of the carbohydrate stores and obligatory use of protein instead. This last possibility seems to be ruled

out by the results of the present work, which show Seckel's effect to be due to increased synthesis of glycogen rather than to prevention of breakdown.

It is impossible to decide from the data at present available whether the extra content of glycogen or of total carbohydrate above the control found in our experiments after incubation in presence of added extract is due in whole or in part to synthesis from protein. With livers rich in glycogen to which no substrate was added, the source might be the glycogen, breaking down in one region and being resynthesized in another; when substrates were added, these might be the source. Light could be thrown on this last question by the use of substrate with labelled carbon. But the extra formation might equally well be due to effect of the active principle upon synthesis from the liver's own protein or protein breakdown products; the fact that it seems to be independent of the particular substrate added perhaps is in favour of this view that the source is endogenous. It is interesting to notice that Villee & Hastings (1949), studying glycogen formation *in vitro* in the diaphragm, have found by use of substrates containing ^{14}C that with glucose only an average of 60 %, with pyruvate only an average of 13 % of the glycogen formed comes from the substrate, the rest from an unknown source. In some of our cases without added substrate, the total carbohydrate after incubation in the presence of extract was greater than the initial value (further examples of this effect after incubation with pure steroids are given in the following paper (Chiu, 1950)) and here the most likely source is protein.

Table 3 summarizes some results, by other workers, on the effect of adrenalectomy and subsequent hormone treatment on the power of tissue slices and of diaphragm fragments to synthesize carbohydrate *in vitro*.

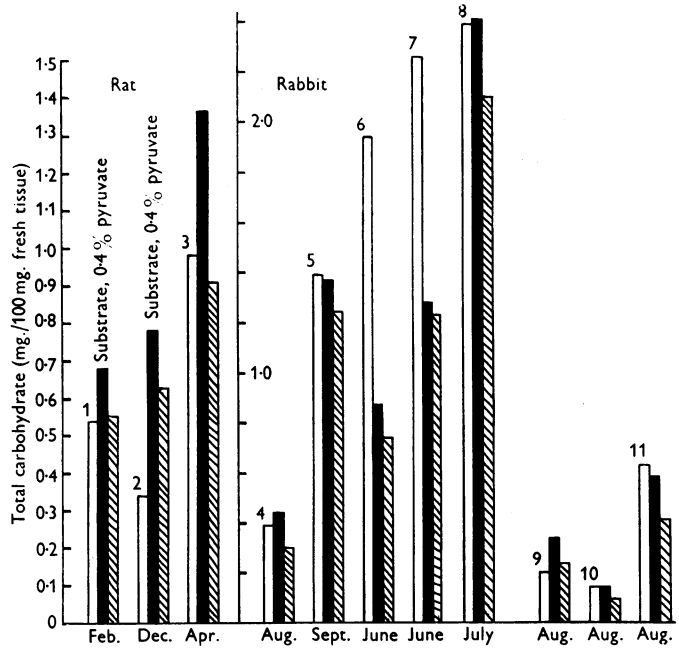

Fig. 3. Total carbohydrate changes in rat- and rabbit-liver slices with and without eschatin. All animals fasted, rats for 24 hr., rabbits for 48 hr. Incubation, 1 hr. at 37°. Medium: phosphate-Ringer for nos. 1, 2 and 8–11; Krebs's Ringer, for nos. 3–5 and 7; bicarbonate-Ringer, for no. 6. Gas phase: O_2 or 95 % O_2–5 % CO_2 (according to the medium). No substrate added, except in 1 and 2. Method (*a*) was used for total carbohydrate estimation for all cases except no 3, where method (*b*) was used. Unshaded = initial value; black = value after incubation with substrate + eschatin; shaded = value after incubation with substrate only.

The results upon synthesis of glycogen from added glucose, where the lowered capacity of the slices after adrenalectomy was very marked, may be complicated by the effect of initial low glycogen content (see above). With the other substrates, the effects of adrenalectomy are varied and cannot be covered by any single simple hypothesis of the action of the hormone. That protein breakdown products may be playing a part is indicated by the very interesting observation of Russell & Wilhelmi (1941*a*) that the rate of deamination of alanine and glutamic acid in kidney slices is decreased some 15 % after adrenalectomy and can be restored to normal value or above by injection into the animal of adrenal cortical

extract or of deoxycorticosterone. Some preliminary experiments indicating that protein may be concerned in carbohydrate synthesis in our experiments are described in the following paper (Chiu, 1950).

It is interesting to notice that White & Dougherty (1947) have concluded, from weight and nitrogen analyses of lymphoid organs, liver and carcass in fasting normal, adrenalectomized, thyroidectomized and adrenalectomized-thyroidectomized mice both corticosterone and corticosterone, the former having greater action than the latter. Riesser (1947) concluded from his similar experiments that deoxycorticosterone and cortin had no effect on glycogen formation.

Amongst other functions which have been suggested for the adrenal cortical hormone is that of activating phosphorylation of carbohydrate. This theory has been put forward by Verzár (see Verzár,

Table 3. *Summary of results by other authors on the effects of adrenalectomy and of adrenalectomy + replacement therapy upon synthetic power of tissue slices*

			Effect of adrenalectomy on synthesis of		Effect of replacement therapy upon synthesis of	
Authors	Tissue	Substrate	Glycogen	Total carbohydrate	Glycogen	Total carbohydrate
Holmes & Lehmann (1940)	Liver (fasted rabbit)	Glucose	Marked decrease	—	—	—
		Lactate	—	No effect	—	—
Bendall & Lehmann (1942)	Liver (fasted rat)	Glucose	Marked decrease	—	Restored to normal	—
Koepf, Horn, Gemmill & Thorn (1941)	Liver (fasted rat)	Lactate	—	No effect	—	Increased above normal
		Pyruvate	—	Approx. 30% decrease	—	Increased above normal
		Glutamate	—	No effect	—	Increased above normal
		Without substrate	—	No effect	—	No effect
Russell & Wilhelmi (1941*b*)	Kidney (rat)	DL-Alanine	—	Approx. 30% decrease	—	—
		L-Glutamate	—	Approx. 30% decrease	—	—
		α-Ketoglutarate	—	Approx. 30% decrease	—	—
		Succinate	—	No effect	—	—
		Pyruvate	—	No effect	—	—
		No substrate	—	No effect	—	—
Villee & Hastings (1949)	Diaphragm (rat)	Glucose	Marked decrease	—	—	—

without and with hormone injections, that the steroid hormones of the adrenal cortex regulate the release of nitrogen from liver and lymphoid tissue while the thyroid secretion controls the rate of nitrogen loss from the liver and carcass. This different reaction of liver and muscle protein to adrenal cortical hormone may be a factor in explaining the very different behaviour as regards glycogen formation of liver and of diaphragm *in vitro* in the presence of added adrenal cortical extract or steroids. Using diaphragm incubated in glucose-containing medium Verzár & Wenner (1948*a*, *c*) and Leupin & Verzár (1949) found decreased glycogen content in the presence of deoxycorticosterone; corticosterone, 17-hydroxycorticosterone and 11-dehydrocorticosterone had a similar but smaller effect. Bartlett, Wick & MacKay (1949) also obtained this lowering of glycogen content with deoxy-

1939); the cases he considers seem to be those where oxidative phosphorylation would be expected, although the theory seems to have been tested *in vitro* only by finding the effect of added adrenal cortical preparations upon phosphorylase activity (see Verzár & Montigel, 1942). We tried a few experiments in which the adenosinetriphosphate level during the incubation with substrate in oxygen was compared in samples with and without eschatin. Values were 9·20 mg. nine-minute phosphorus/100 g. tissue without and 9·25 mg. with extract; in another experiment, 12·0 and 12·9 mg. without and with hormone, respectively. Thus no effect was found, but such experiments cannot be conclusive. Possibly, by means of radioactive phosphate, any difference in the rate of turnover of the adenylic compounds in presence of adrenal cortical preparations could be detected.

SUMMARY

1. The action of adrenal cortical extract (added *in vitro*) in causing diminution of glycogen breakdown in liver slices (rat and rabbit) has been confirmed.

2. It was shown that this inhibition is absent if the incubation of the liver slices is carried out in absence of oxygen. It is concluded that the active principle affects the synthesis rather than the breakdown of glycogen.

3. When total carbohydrate was estimated, a similar effect of the extract in preventing disappearance of the carbohydrate was observed. The effect is, therefore, not merely on the glycogen: glucose ratio.

4. Under conditions where carbohydrate synthesis can be demonstrated, there was always greater increase (whether estimated as glycogen or total carbohydrate) in the presence of the extract.

5. No effect was found on the adenosinetriphosphate level in the slices during carbohydrate synthesis in oxygen.

We are very grateful to Prof. D. Keilin, F.R.S., Dr E. F. Hartree and Prof. E. A. Doisy for gifts of glucose oxidase and catalase, and to Dr T. Mann for help in preparing glucose oxidase; we are greatly indebted to Messrs Parke, Davis and Co. for the supplies of eschatin free from preservative. Our thanks are due to the British Council for a studentship (C.Y.C.) and to the Medical Research Council for a personal grant (D.M.N.).

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The Effect of Adrenal Cortical Preparations added *in vitro* upon the Carbohydrate Metabolism of Liver Slices

2. THE EFFECT OF SOME PURE STEROIDS UPON CARBOHYDRATE SYNTHESIS, OXYGEN UPTAKE AND NON-PROTEIN NITROGEN

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(*Received* 15 *August* 1949)

After it had been shown that addition of adrenal cortical extract *in vitro* caused increased glycogen and total carbohydrate formation in liver slices (Chiu & Needham, 1950), it became of interest to find the effect of some pure adrenal cortical steroids. Deoxycorticosterone (DOC, as acetate and hydrogen succinate), 11-dehydrocorticosterone and 17-hydroxy-11-dehydrocorticosterone became available, and experiments are now described on their action, particularly in causing increased total carbohydrate synthesis. Some observations were also made on accompanying oxygen uptake and, in order to see whether participation of protein in the synthesis was indicated, on the changes in non-protein nitrogen.