Effects of Adrenal Corticoids on Fatty Acid Synthesis in Mammary Gland Slices *in vitro*

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(Received 16 September 1954)

The antagonism between insulin and the adrenal glucocorticoids in intermediary metabolism is well known; in the whole animal it expresses itself by the antidiabetogenic action of insulin on the one hand and the diabetogenic action of the adrenal cortex on the other. The mechanism of such hormone interactions will be elucidated only when it is possible to study them with isolated enzyme systems, or at least with the intact cell. As a first step it is of interest to see if they can be demonstrated in isolated tissues and then perhaps in cell-free preparations.

A well-authenticated effect of insulin on isolated tissues *in vitro* is its ability to accelerate the rate of fatty acid synthesis from C_2 units arising from acetate or glucose. This was first studied in rat liver slices (Bloch & Kramer, 1948; Brady & Gurin, 1950), but is particularly marked in slices of mammary gland from lactating rats (Balmain, French & Folley, 1950; Balmain & Folley, 1951; Balmain, Folley & Glascock, 1951, 1952a, 1954a), a tissue which provides an eminently useful medium for studying fatty acid synthesis *in vitro*. Irrespective of the mechanism by which insulin promotes fatty acid synthesis under these conditions, the antagonism of insulin and adrenal corticoids might be expected to manifest itself at some point in the pathway of fatty acid synthesis, which would imply a direct or indirect role for the adrenal cortex in the regulation of fatty acid metabolism.

Very little work on the relationship between the adrenal cortex and fatty acid metabolism has, so far, been done with tissue slices. Brady, Lukens & Gurin (1951) reported that liver slices from rats pre-treated with cortisone show a diminished incorporation of acetate (carboxy) carbon into fatty acids. In line with this, we have shown in preliminary work that cortisone, added to the incubation medium, inhibits fatty acid synthesis from acetate in slices of mammary gland from rats killed at the end of pregnancy, in early lactation (days 1-4) or in full lactation. In fully lactating rat mammary tissue, which, unlike tissue taken at the end of pregnancy, is sensitive to the action of insulin in promoting the synthesis of fatty acids *in vitro*, cortisone inhibited the insulin effect (Balmain *et al.* 1952b). The present paper reports further experiments on the effect of cortisone on fatty acid synthesis in rat mammary

slices and extends the work to other adrenal steroids and to udder tissue from ruminants (sheep). A preliminary account of some of these experiments has already been published (Balmain, Folley, Glascock & McNaught, 1954b).

EXPERIMENTAL

Tissue. Experiments on rats were carried out on abdominal mammary tissue from primiparous hooded Norway rats killed at days 11-16 of lactation. For most experiments the tissue from three rats was pooled, and slices prepared as described previously (Balmain *et al.* 1952a). In a few experiments the tissue from individual rats was divided between treatment and control (see Table 2). For experiments on sheep, tissue was obtained from lactating primiparous ewes slaughtered at 21-33 days after parturition. Separate experiments were done with the tissue from the two halves of each udder.

Incubation technique. Batches of slices weighing 1-2 g. were suspended in saline medium (10 ml./g. tissue) in large Warburg flasks fitted with manometers. The flasks were gassed with a 5% CO_2 + 95% O_2 mixture for bicarbonate saline or 100% O_2 for phosphate-saline experiments and then incubated for 2-3 hr. at 37° with shaking. In each experiment a 'blank' flask was set up in which the tissue was killed at zero time.

Medium. The medium was prepared by dissolving the substrates, glucose (0.3%, w/v) and sodium acetate (0.02M), in saline (Krebs & Henseleit, 1932) buffered with bicarbonate, save in three experiments (see Table 2) in which it was buffered with phosphate. In some experiments the substrates were sodium [carboxy- ^{14}C]acetate + unlabelled glucose, in others sodium [α - 3H]acetate + [^{14}C]glucose.

Addition of hormones. The insulin used throughout was a crystalline preparation (22 i.u./mg.) free from glucagon; it was added to the incubation medium at a final concentration of 1.0 $\mu g./ml.$ except in one experiment (see Table 1) in which the concentration was 0.045 $\mu g./ml.$ In two of the earlier experiments (Tables 1 and 3) crystalline cortisone (free alcohol) was shaken at 37° with the medium overnight before the experiment and the undissolved steroid shaken into suspension before pipetting. Later, cortisone and other adrenal steroids were added to the medium in acetone solution (10 mg./ml.), corresponding amounts of acetone being added to the medium used in control incubations. We believe that under these conditions the steroids remained in solution, since no turbidity whatever developed when the acetone solutions were added to the medium, and we regularly observed graded concentration/response relationships.

Isolation of fatty acids. At the end of the incubation period the tissue was killed by addition of HCl to a concentration of 0.3N. The contents of the flasks were then evaporated to dryness under reduced pressure and saponified for 24 hr. with ethanolic KOH (5 ml. 40% (w/v) KOH + 10 ml. ethanol). The free fatty acids were extracted and titrated with $\text{Ca}(\text{OH})_2$ as previously described (Balmain *et al.* 1952a).

Determination of isotopes. The Ca salts were assayed for ^{14}C either by the gas-counting method of Glascock (1952) or by counting at infinite thickness on 1 cm.² disks with a thin mica-window counter, the two counting methods being cross-calibrated. In experiments in which tritioacetate was used as substrate, tritium was assayed as tritio-butane (Glascock, 1951). In Tables 1-7, results of experiments other than those involving tritium were obtained by end-window counting, except where otherwise stated. In all experiments the values for the 'blank' flask, in which the tissue was killed at zero time, were negligible.

RESULTS

Cortisone

Rat. The results of five experiments are shown in Table 1. In confirmation of the single experiment reported in a preliminary communication (Balmain *et al.* 1952b) and included in this table, it will be seen that in the presence of cortisone (approx. 100 $\mu\text{g./ml.}$) the incorporation of glucose carbon into the mammary gland fatty acids was reduced to

57-85% of the control value, and the incorporation of acetate (methyl) carbon was reduced to 27-85%. It is clear therefore that insulin and cortisone are capable of exerting mutually antagonistic effects on fatty acid synthesis. The results of five further experiments in which cortisone (100 $\mu\text{g./ml.}$ final concentration) was added to the medium in acetone solution are shown in Table 2. In all these experiments, which included three in which the incubation medium was phosphate-saline and the gas phase pure O_2 , cortisone inhibited fatty acid synthesis from acetate by the mammary gland slices, though in the experiment shown in the third column, the inhibition was hardly significant.

Sheep. The results of six experiments on tissue from three lactating ewes are given in Table 3. Sheep udder tissue does not utilize glucose very actively for fatty acid synthesis *in vitro*, consequently the incorporation of glucose carbon (^{14}C) was in all cases rather low as compared with rat mammary tissue (Balmain *et al.* 1954a). The results provide no evidence that cortisone, in a much higher concentration than used in the rat experiments, significantly affected the incorporation of glucose carbon either way. Neither do the tritium specific activities show any consistent and significant effects of cortisone on lipogenesis from acetate.

Table 1. *Effect of cortisone and insulin, in vitro, on fatty acid synthesis from glucose and acetate in mammary gland slices from lactating rats*

The substrates were 0.3% [^{14}C]glucose (3.3 $\mu\text{mc/mg.}$) + 0.02M-Na [α - ^3H]acetate (30.5 $\mu\text{mc/mg.}$).

Addition	Expt. no.					Mean (as % of control)
	B 256	B 257	B 258	B 261	B 262	
¹⁴ C (glucose carbon) in fatty acids (counts/min./mg. C)						
None	366	486	485	312	347	100
Cortisone (approx. 100 μg./ml.)	254	275	403	265	219	71
Insulin (0.045 μg./ml.)	795	833	928	533	570	183
Cortisone + insulin	383	—	488	348	325	97
³ H (methyl carbon of acetate) in fatty acids (counts/min./mg. combustion water)						
None	283	168	301	279	299	100
Cortisone (approx. 100 μg./ml.)	77	96	170	236	175	57
Insulin (0.045 μg./ml.)	430	308	682	499	410	175
Cortisone + insulin	118	—	383	254	214	73

Table 2. *Effect of cortisone in vitro on fatty acid synthesis from glucose in mammary gland slices from lactating rats*

The substrates were 0.3% [^{14}C]glucose (16.5 $\mu\text{mc/mg.}$) + 0.02M-Na [α - ^3H]acetate (30.5 $\mu\text{mc/mg.}$). Tritium was not assayed. For rats 42147, 42116 and 42102 substrates were dissolved in phosphate-saline, and for the other two rats bicarbonate-saline was used. ^{14}C specific activities expressed as counts/min./mg. C of the Ca salts of the mixed fatty acids.

Rat no. ...	42147	42116	42102	42459	42476	Mean (as % of control)
Day of lactation ...	15	13	16	12	12	
Control	680	252	686	1350	1980	100
Cortisone (100 $\mu\text{g./ml.}$)	323	85	625	980	1605	73

Table 3. *Effect of cortisone, added in vitro, on fatty acid synthesis from glucose and acetate in udder slices from lactating ewes*

The substrates were 0.3% [^{14}C]glucose (3.5 $\mu\text{mc/mg.}$) + 0.02M-Na [$\text{carboxy-}^{14}\text{C}$, $\alpha\text{-}^3\text{H}$]acetate (13 $\mu\text{mc/mg.}$). ^{13}C was not assayed.

Udder half no.	...	...	...	S4L	S4R	S5L	S5R	S6L	S6R	Mean (as % of control)
^{14}C (glucose carbon) in fatty acids (counts/min./mg. C)										
Control				38.4	17.4	22.5	24.7	35.8	31.6	100
Cortisone (approx. 250 $\mu\text{g./ml.}$)				32.6	34.8	24.3	19.8	37.0	23.2	101
^3H (methyl carbon of acetate) in fatty acids (counts/min./mg. combustion water)										
Control				843	810	1002	1004	903	1004	100
Cortisone (approx. 250 $\mu\text{g./ml.}$)				833	895	895	810	953	983	96

Table 4. *Effect of cortisone in vitro on fatty acid synthesis from acetate in udder slices from lactating ewes*

The substrates were 0.3% glucose + 0.02M-Na [$\text{carboxy-}^{14}\text{C}$]acetate (30.5 $\mu\text{mc/mg.}$). ^{14}C specific activities expressed as counts/min./mg. C of Ca salts of mixed fatty acids.

Concentration of cortisone ($\mu\text{g./ml.}$)	Expt. no.			
	S9R	S9L	S10R	S10L
0	8470	8580	13 875	12 475
50	8850	—	15 150	—
100	—	7500	—	12 100

The results of four further experiments, involving smaller concentrations of cortisone, carried out on tissue from the separate udder halves of two lactating ewes are shown in Table 4. Here again there is no evidence of any consistent inhibitory effect of cortisone on fatty acid synthesis from acetate.

Cortisol (hydrocortisone)

Rat. Table 5 gives the results of five experiments on mammary slices from lactating rats in which three concentrations of cortisol were studied, two of them in the presence and absence of insulin. It is

quite clear that, unlike cortisone, cortisol in concentrations up to 100 $\mu\text{g./ml.}$ had no inhibitory effect on fatty acid synthesis from glucose either in the presence or absence of insulin; on the contrary, the results suggest that cortisol, if anything, slightly increased the rate of fatty acid synthesis from glucose in the slices. In order to investigate this possibility still further, two experiments were set up each involving pooled mammary slices from three rats, the substrate being [^{14}C]glucose + [$\alpha\text{-}^3\text{H}$]acetate. From each pool of slices ten flasks were prepared in the usual way, five of which contained 100 $\mu\text{g.}$ cortisol/ml. medium. The results given in Table 6 show little evidence of any significant stimulation by cortisol. On the other hand, the fact that cortisol does not in any way inhibit fatty acid synthesis has been amply confirmed. Two experiments, similar in all respects, save that insulin (1.0 $\mu\text{g./ml.}$) was present in all flasks, were carried out. The results, also given in Table 6, indicate that cortisol in a concentration of 100 $\mu\text{g./ml.}$ did not significantly affect the stimulating effect of insulin, at the concentration used, on fatty acid synthesis from acetate and glucose.

Table 5. *Effect of cortisol, corticosterone and 11-deoxycorticosterone (DOC), alone and with insulin, in vitro on fatty acid synthesis from glucose in mammary gland slices from lactating rats*

The substrates were 0.3% [^{14}C]glucose (16.5 $\mu\text{mc/mg.}$) + 0.02M-Na [$\alpha\text{-}^3\text{H}$]acetate (30.5 $\mu\text{mc/mg.}$). Tritium was not assayed. ^{14}C specific activities expressed as counts/min./mg. C of Ca salts of mixed fatty acids.

Expt. no.	...	...	...	RS1		RS2		RS3		RS4	RS5	RS10*	RS11*
Insulin concentration ($\mu\text{g./ml.}$)				0	1.0	0	1.0	0	1.0	0	0	0	0
Steroid added ($\mu\text{g./ml.}$)													
Control	0			2665	4210	2300	4540	3100	4475	2445	2103	2358 $\pm$ 141	3088 $\pm$ 35
Cortisol	50			—	—	—	—	3400	3900	2448	2690	—	—
	75			—	—	—	—	—	—	2819	2720	—	—
	100			2802	3720	3620	5200	—	—	2813	2475	—	—
Corticosterone	50			—	—	—	—	2120	3980	—	—	—	—
	100			1016	1531	675	1200	—	—	—	—	588 $\pm$ 61	1114 $\pm$ 88
DOC	50			—	—	—	—	2940	4150	2203	—	—	—
	75			—	—	—	—	—	—	—	1843	—	—
	100			581	860	480	815	—	—	—	—	661 $\pm$ 79	954 $\pm$ 77

* Four replicate flasks.

Table 6. *Effect of cortisol alone and with insulin in vitro on fatty acid synthesis from glucose and acetate in mammary gland slices from lactating rats*

Mean values and standard errors of the specific activities of the Ca salts of the fatty acids. The substrates were 0.3% [^{14}C]glucose (16.5 $\mu\text{mc}/\text{mg.}$) + 0.02 M-Na [α - ^3H]acetate (30.5 $\mu\text{mc}/\text{mg.}$).

Expt. no.	Additions		^{14}C (glucose carbon) (counts/min./mg. C)	^3H (methyl carbon of acetate) (counts/min./mg. combustion water)
	Cortisol ($\mu\text{g.}/\text{ml.}$)	Insulin ($\mu\text{g.}/\text{ml.}$)		
R.S. 6	0	0	3266 $\pm$ 136	658 $\pm$ 44
	100	0	3466 $\pm$ 37	696 $\pm$ 19
R.S. 7	0	0	3608 $\pm$ 79	662 $\pm$ 37
	100	0	4017 $\pm$ 101	633 $\pm$ 32
R.S. 8	0	1.0	3394 $\pm$ 137	495 $\pm$ 23
	100	1.0	3670 $\pm$ 157	482 $\pm$ 25
R.S. 9	0	1.0	3651 $\pm$ 143	329 $\pm$ 25
	100	1.0	3498 $\pm$ 376	*352 $\pm$ 42

* Mean of 4, others of 5.

Table 7. *Effect of cortisol, corticosterone and 11-deoxycorticosterone (DOC) in vitro on fatty acid synthesis from acetate in udder slices from lactating ewes*

The substrates were 0.3% glucose + 0.02 M-Na [*carboxy*- ^{14}C]acetate (30.5 $\mu\text{mc}/\text{mg.}$). ^{14}C specific activities expressed as counts/min./mg. C of Ca salts of mixed fatty acids.

Steroid added ($\mu\text{g.}/\text{ml.}$)	Expt. no.					
	S8R	S8L	S9R	S9L	S10R	S10L
Control 0	3400	2650	8470	8580	13 875	12 475
Cortisol { 50 100	—	—	8650	—	14 150	—
	2240	1670	—	8100	—	12 550
Corticosterone { 50 100 200	—	—	7320	—	12 450	—
	835	776	—	6560	—	9740
	173	173	—	—	—	—
DOC { 50 100 200	—	—	8720	—	10 600	—
	740	800	—	4720	—	5200
	85	115	—	—	—	—

Sheep. Table 7 shows the results of experiments in which the effect of cortisol on fatty acid synthesis from acetate was studied in six experiments on udder tissue from three lactating ewes. In two of the experiments (nos. S9L and S10L), involving 100 $\mu\text{g.}$ cortisol/ml. medium, on tissue from two of the ewes, cortisol had no significant effect on the rate of fatty acid synthesis from acetate, but in the two experiments on tissue from the separate udder halves of the third sheep, cortisol at this concentration significantly inhibited the incorporation of acetate carbon into the fatty acids. In the two experiments in which cortisol was present at a concentration of 50 $\mu\text{g.}/\text{ml.}$, the steroid exerted no inhibitory action.

Corticosterone

The results given in Tables 5 and 7 show quite unequivocally that corticosterone (100 $\mu\text{g.}/\text{ml.}$) markedly inhibits fatty acid synthesis from glucose in rat mammary slices and from acetate in udder slices from the ewe. In sheep tissue in presence of a concentration of 50 $\mu\text{g.}/\text{ml.}$ the effect was not very

marked, but 200 $\mu\text{g.}/\text{ml.}$ almost completely abolished fatty acid synthesis. Insulin appeared partially to overcome the inhibitory action of corticosterone (100 $\mu\text{g.}/\text{ml.}$) in the rat.

11-Deoxycorticosterone

As Tables 5 and 7 show, 11-deoxycorticosterone (100 $\mu\text{g.}/\text{ml.}$) markedly inhibits fatty acid synthesis in lactating mammary tissue, both from the rat (glucose carbon) and the sheep (acetate carbon). The effects of lower concentrations of steroid though evident, were not so marked. In the sheep, 200 $\mu\text{g.}/\text{ml.}$ virtually abolished fatty acid synthesis. Insulin to a small extent overcame the inhibition of fatty acid synthesis from glucose in rat mammary slices caused by 100 $\mu\text{g.}/\text{ml.}$ deoxycorticosterone.

DISCUSSION

The work described in this paper has confirmed our previous preliminary observations on the inhibitory action of cortisone on the rate of fatty acid synthesis

from glucose and acetate in mammary gland slices from lactating rats. It has also been shown that the cortisone inhibition can be overcome completely or almost so by addition of insulin to the medium. Our results indicate that the two hormones are mutually antagonistic in respect of their modulating effects on fatty acid synthesis in rat mammary tissue, as might be expected from their physiological activities *in vivo*. As regards fatty acid synthesis, insulin exerts an anabolic effect (just as it does in the diabetic animal) and cortisone a catabolic one. This does not apply to ruminant mammary tissue as exemplified by the sheep, for which our results provide no evidence of an inhibitory effect of cortisone on fatty acid synthesis. Neither, as our previous results show, is fatty acid synthesis in sheep udder tissue sensitive to the stimulatory action of insulin (Balmain & Folley, 1951; Balmain *et al.* 1952*a*, 1954*a*). Since we have shown that sheep udder slices, under similar conditions to the present experiments, utilize practically no glucose for fatty acid synthesis, while rat mammary gland slices use glucose and acetate about equally (Balmain *et al.* 1954*a*), it seems likely that the above-mentioned opposing actions of insulin and cortisone are exerted at some point along the route of utilization of glucose for fatty acid synthesis. Against this, it

must be remembered that insulin increases the incorporation not only of glucose carbon but also of acetate carbon into the fatty acids of rat mammary gland (Balmain *et al.* 1954*a*) and conversely, as shown in the present work, cortisone inhibits the utilization of acetate as well as glucose carbon.

Our results show that there are certain differences between various adrenal corticoids as regards their effects on fatty acid synthesis in mammary gland slices. At the same concentration (100 $\mu\text{g./ml.}$) at which cortisone exerts definite inhibitory effects on rat mammary gland slices, cortisol shows no inhibitory effect whatever either in the presence or absence of insulin. In fact some of our results suggest that cortisol might exert a slight stimulatory effect on fatty acid synthesis from glucose. Its effects on sheep udder tissue were not clear-cut, a slight inhibitory effect being observed in some experiments but not in others. On the other hand, at the same concentration, corticosterone and 11-deoxycorticosterone inhibit fatty acid synthesis in rat mammary tissue much more strongly than does cortisone, and also exert marked inhibitory effects on sheep mammary slices, which are inert to cortisone. The inhibitory effects on fatty acid synthesis of the four steroids studied are summarized in a roughly quantitative manner in Table 8, which

Table 8. *Relative inhibitory effects of adrenal corticoids in vitro on fatty acid synthesis in lactating mammary gland slices*

Compound	Structural formula	Inhibitory effect in tissue from	
		Rat	Sheep
Cortisol (compound F)		0	+?
Cortisone (compound E)		+	0
Corticosterone (compound B)		+++	++(+++?)
11-Deoxycorticosterone		+++	+++

'+' Indicates degree of effect.

shows that the four compounds fall into two classes, comprising on the one hand, cortisol and cortisone, which at the concentration studied either have no inhibitory effects or only moderate ones and on the other, corticosterone and 11-deoxycorticosterone, which at the same concentration are much more strongly inhibitory. Consideration of the structural formulae of the four steroids suggests the possibility that the 17-hydroxy group tends to suppress the inhibitory effect of the steroid upon fatty acid synthesis.

The action of adrenal corticoids in inhibiting fatty acid synthesis in mammary tissue is reminiscent of their action in inhibiting the net synthesis of glycogen in the isolated rat diaphragm reported by Verzář & Wenner (1948*a*, *b*) and Leupin & Verzář (1950). The effective concentrations reported by these authors (10–100 $\mu\text{g./ml.}$) were somewhat lower than those found by us. However, they found that whereas 11-deoxycorticosterone was very active, those corticoids with an 11-hydroxyl group (corticosterone and 17-hydroxycorticosterone) were much less active. Results similar in many respects were briefly reported by Grossman, Ryder & Pearson (1953). Thus the behaviour of corticosterone as regards glycogen deposition in the rat diaphragm and fatty acid synthesis in mammary tissue is very different. This does not necessarily mean that the same primary effect (e.g. an action on the permeability of the cell to substrates) is not involved in the two cases, for the tissues studied are different. Using rat liver slices, Chiu & Needham (1950) and Chiu (1950) found that, by contrast with the results obtained with the rat diaphragm cited above, equilibration with adrenal corticoids (cortisone, 11-dehydrocorticosterone or 11-deoxycorticosterone) increased glycogen synthesis. They attributed the difference between their findings and those of Verzář & Wenner on diaphragm to the fact that different tissues were involved. However, the possibility that corticoids may exert opposite effects at different concentrations must be kept in mind. In some experiments in our laboratory (Balmain, Folley & Glascock, unpublished) we have found that relatively low concentrations of corticosterone (*ca.* 10 $\mu\text{g./ml.}$) stimulated fatty acid synthesis from small molecules in rat mammary gland slices, while higher concentrations inhibited this process as found in the present work. However, we have not yet succeeded in reproducing these results regularly.

The question arises whether the present findings have any bearing on physiological processes in the living animal. Such information as we have on the concentration of corticoids in peripheral blood (e.g. Nelson & Samuels, 1952) suggests that the normal concentrations are very much smaller than 100 $\mu\text{g./ml.}$ —perhaps by a factor of 10^3 . We are not

therefore prepared to assert that these results are of physiological significance, although in view of the different conditions obtaining *in vivo* and *in vitro* we feel that it would be unwise to say they are not.

In respect of the action of cortisone on lipogenesis *in vitro* our present results provide another example of a difference between the metabolic behaviour *in vitro* of mammary tissue from non-ruminants, exemplified by the rat, and from ruminants, represented by the sheep. The significance of this species difference and its relation to other species differences in mammary tissue metabolism observed in our laboratory (Folley & French, 1949, 1950; Balmain & Folley, 1951; Balmain *et al.* 1952*a*, 1954*a*; Duncombe & Glascock, 1954), needs further investigation.

SUMMARY

1. The effect of cortisone, cortisol, corticosterone and deoxycorticosterone, alone and in conjunction with insulin, on the incorporation of glucose and acetate labelled with ^{14}C or ^3H into the fatty acids of lactating mammary gland slices from rats and sheep has been studied.

2. In rat tissue cortisone at a concentration of 100 $\mu\text{g./ml.}$ medium was found to reduce fatty acid synthesis from glucose to approximately 70 % of the control value and from acetate to approximately 60 % of the control; the stimulating effect of insulin (0.045 $\mu\text{g./ml.}$) was largely antagonized by the cortisone addition. With sheep tissue, 100 $\mu\text{g./ml.}$ and even 250 $\mu\text{g./ml.}$ cortisone failed to reduce fatty acid synthesis from acetate.

3. Cortisol at 100 $\mu\text{g./ml.}$ had no significant effect on the incorporation of either acetate or glucose carbon into fatty acids in rat tissue, nor did it antagonize the stimulation produced by 1.0 $\mu\text{g./ml.}$ insulin: in sheep tissue there was some evidence that it could inhibit the synthesis of fatty acids from acetate.

4. Corticosterone at 100 $\mu\text{g./ml.}$ markedly inhibited fatty acid synthesis from glucose in rat tissue and reduced the stimulating effect of 1.0 $\mu\text{g./ml.}$ insulin. In sheep tissue, 50 $\mu\text{g./ml.}$ slightly inhibited fatty acid synthesis from acetate, 100 $\mu\text{g.}$ caused pronounced inhibition and at 200 $\mu\text{g./ml.}$ fatty acid synthesis was almost suppressed.

5. Deoxycorticosterone behaved very similarly to corticosterone, but was slightly more inhibitory in sheep at 100 $\mu\text{g./ml.}$ than corticosterone.

6. These findings are discussed in relation to species differences known to exist between ruminant and non-ruminant tissue and to the structural formula of the steroids.

We are grateful to Dr C. C. Porter of the Merck Institute for Therapeutic Research, Rahway, New Jersey, for cortisone (free alcohol), to Dr A. White, then of Chemical

Specialities (Syntex) Co. Inc., New York City, for corticosterone and hydrocortisone; and to Dr W. J. Tindall of Organon Laboratories Ltd. for deoxycorticosterone. Dr K. Hallas-Møller of the Novo Terapeutisk Laboratorium, Copenhagen, kindly gave us generous supplies of crystalline insulin free from glucagon. The technical assistance of Messrs S. C. Watson and B. W. E. Peaple is much appreciated, and we are indebted to Miss May Beard for the care of the sheep.

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Cacao Polyphenolic Substances

3. SEPARATION AND ESTIMATION ON PAPER CHROMATOGRAMS*

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(Received 8 October 1954)

Previous work on the chromatography of cacao polyphenols was carried out on one-way chromatograms with amyl alcohol-acetic acid, *n*-butanol-acetic acid, or phenol (Forsyth, 1952a). It was shown that the main polyphenols present could be grouped as cyanidin glycosides, catechins and the so-called 'leucoanthocyanins'. The introduction of water as a solvent for the separation of polyphenols on a paper chromatogram (Roberts & Wood, 1953) has greatly assisted the study of the cacao polyphenols, especially the ill-defined 'leucoanthocyanin' group. A previous attempt (Forsyth, 1952b) to estimate the polyphenols on the chromatograms has been greatly improved. Williams (1953) has applied a similar method to the tannin of cider.

MATERIALS AND METHODS

Imperial College of Tropical Agriculture Selection Clone no. 1 beans were used throughout.

Dehydrated cotyledons. Great care must be taken in the drying of cacao beans because of the powerful oxidase systems present. Fortunately the enzymes are spatially separated from the polyphenols in the fresh bean. De-

hydrated bean powder can therefore be prepared (Forsyth, 1952b) with the phenolic components unchanged (Forsyth & Rombouts, 1952).

Polyphenol storage cells. The cells containing the polyphenols were separated from powdered dried cotyledons by sedimentation in light petroleum (b.p. 80–100°) by the method of Brown (1954). It is preferable to use a long shallow trough, tilted at a slight angle with the light petroleum flowing gently over the powder. In this way a heavy fraction containing only purple cells (97% soluble in 70% (v/v) aqueous acetone) was obtained. These cells were free from polyphenol oxidase which could be recovered from a lighter fraction (Forsyth & Quesnel, unpublished.)

Ethyl acetate soluble polyphenols. Freshly dried cacao cotyledons (1 kg.) were blended in small lots in a Waring Blendor with parts of 8 l. of 0.1N-HCl cooled to 0°. After blending, the bulk of the 0.1N-HCl, cooled to 0°, was added and the suspension stirred for 15 min. The suspension was then filtered through muslin and cleared in a Sharples centrifuge. The acid extract (ca. 6 l.) was saturated with NaCl and stirred for 15 min. with ethyl acetate (3 l.). The mixture was separated by passing through a Sharples centrifugal separator and the aqueous layer again treated with ethyl acetate (1.5 l.). The combined ethyl acetate extracts were dried over Na₂SO₄ (150 g.) containing NaHCO₃ (50 g.). Because of the known instability of the leucocyanidins in acid solution (Forsyth, 1953) no delay was warranted up to this stage. Thereafter the ethyl acetate

* Part 2: Forsyth (1952b).