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Review

Pharmacology and Biochemistry of some Amphenone Analogues and other Adrenal Cortical Inhibitors*

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Introduction

The acquisition of a greater understanding of the relationship between adrenal cortical function and health and disease has indicated clearly that alteration of this function could, under proper circumstances, be of great value in clinical medicine. The preparation of ACTH suitable for clinical use as a means of stimulating cortical function and its impact on modern medicine is a well documented story. On the other hand, a practical solution to the problem of suppressing adrenal cortical function has not been forthcoming, but since the 1940s, when it was first reported that suramin sodium[1] induced what appeared to be a suppression of the adrenal cortex, progress has been achieved.

A compound which could induce a generalized inhibition of cortical function or a series of compounds which could inhibit selectively various aspects of cortical function would be of obvious value in treating the different syndromes of adrenal cortical hyperfunction. Fortunately, by clinical standards, the incidence of Cushing's syndrome, the adrenogenital syndrome and primary aldosteronism is relatively rare.

A much broader application for compounds of this sort would be found in conditions involving secondary hyperfunction of the adrenal cortex, e.g. secondary aldosteronism. The work of many investigators indicating that hypersecretion of aldosterone

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accompanies congestive heart failure, hepatic cirrhosis, the nephrotic syndrome, and normal and toxaemic pregnancies has been reviewed adequately.[2–4] Present evidence concerning the hypersecretion of aldosterone in these conditions indicates that the mechanisms are highly complex and are still somewhat obscure. In view of the fact that water and salt retention are common to all these conditions, it is self-evident that inhibition of aldosterone at its source and the induction of natriuresis could be highly desirable. If sodium is excreted, water might be expected to follow.

Inhibition of Normal Function

There are also certain diseases which may not necessarily entail co-existent adrenal cortical hyperfunction, but nevertheless could be benefited by a reduction in corticoid secretion.

The fact that corticoids of the hydrocortisone type are instrumental in the formation of glucose from non-carbohydrate sources indicates that theoretically it may be of advantage to reduce this secretion or change its pattern in diabetes mellitus.

The suppression of corticoid production to induce sodium excretion in hypertension could be of therapeutic value. This theory finds some support in adrenalectomy studies.[5, 6] Also there is evidence that certain types of cancer are to some extent dependent upon the sex hormone-like secretion of the adrenal cortex.

It is, therefore, feasible to postulate that inhibition of the adrenal may be of benefit in these conditions—again as implied by adrenalectomy studies.[7, 8]

Surgical Inhibition of the Adrenal Cortex

Adrenalectomy

Various means of inhibiting the adrenal cortex have been tried. Surgical removal is the most definitive method, but has its drawbacks, one being that it is essentially an all or none process. In most cases some residual adrenal function would be desirable and this has been attempted by removing one gland *in toto* and 90 per cent of the other. In some instances this remnant is sufficient to take care of the daily corticoid requirements, but in general there is never enough to cope with emergencies; therefore therapy with

exogenous corticoids is necessary in most cases. On the other hand, re-generation of the remnant has been reported.[6] Also many patients are poor surgical risks for major surgery of this nature.

Hypophysectomy

Suppression of adrenal cortical function can, of course, be accomplished by hypophysectomy. This has been demonstrated in man by Luft *et al.*[9] in the treatment of metastatic carcinoma of the breast and diabetes mellitus; Lipsett *et al.*[10] in the treatment of metastatic cancer; and by Gordon[11] in the treatment of diabetic nephropathies. The use of this procedure for the specific purpose of suppressing adrenal function, however, has the disadvantage of upsetting the whole endocrine system by producing panhypopituitarism. The problems of therapy involved in treating this condition must be weighed in each individual case against the possible benefits derived from depressed adrenal function.

Chemotherapeutic Suppression of Cortical Function

Corticoids

The reciprocal relationship between the adrenal cortex and anterior pituitary with respect to ACTH and corticoid secretion, as elucidated by Sayers[12] and Ingle,[13] suggested that administration of small doses of active corticoids might suppress endogenous corticoid secretion. The efficacy of this treatment is most clearly shown in the studies by Wilkins[14] where small doses of cortisone reduced the output of 17-ketosteroids *via* the inhibition of pituitary ACTH in patients with congenital adrenal hyperplasia.

This treatment is of less value in Cushing's syndrome. The more potent pituitary inhibitors are corticoids of the cortisol type and in the Cushing patient one is faced with the problem of administering such a corticoid in the face of already high circulating levels. Also this form of therapy is ineffective in the treatment of primary or secondary aldosteronism. Inhibition of ACTH has relatively little influence on aldosterone secretion.

The use of corticoids to suppress adrenal function assumes that the site of the difficulty lies at the pituitary level. Exogenous corticoid therapy is completely ineffective if cortical hyperfunction

is due to an autonomously functioning adrenal tumour, which often is the case.

Agents with CNS Activity

Various other drugs have been reported to suppress adrenal cortical function by interfering at some point in the pituitary–adrenal system. Ohler and Sevy[15] have shown that morphine and phenoxybenzamine can block adrenal cortical activity in response to an acute stress. Wells, Briggs and Munson[16] observed that an initial dose of reserpine causes adrenal stimulation but repeated doses will not stimulate and furthermore will block the adrenal response to agents which formerly stimulated cortical function. This has been confirmed under other circumstances, such as anxiety states[17] and in schizophrenic patients.[18] Chlorpromazine has also been reported to alter corticoid secretion but the results of various studies appear to be conflicting. Acute studies[19, 20] show stimulation of the adrenal cortex following chlorpromazine, while measurements of cortical function after chronic administration, or at least two hours after chlorpromazine, show a suppression of adrenal function.[21–23]

It is presumed that these agents act in the central nervous system and interfere with the release mechanism for ACTH.

Thiouracil

Thiouracil, a strong antithyroid compound, was reported by Glock[24] in 1946 to cause adrenal atrophy. It was assumed by various investigators that this involution was merely a reflection of a generalized inhibition of body growth.[25, 26] Zarrow *et al.*[27] have recently separated the two factors and find that adrenal inhibition is still evident in animals whose growth has been maintained with vitamin B_{12}. The mechanisms of this inhibition are speculative.

Estrogens

Certain estrogens have been shown to inhibit adrenal cortical function in the rat. Vogt[28] and Holzbauer[29–32] have shown that both ethinyl estradiol and hexestrol cause adrenal hypertrophy, increase adrenal lipid, and reduce corticoid secretion in the maximally stimulated gland. Interference with cholesterol

synthesis is postulated as a mechanism. The impracticality of such compounds as adrenal inhibitors in man is obvious, however, when one takes into account their feminizing activity. Nevertheless, Taliaferro *et al.*[33] have tested stilbestrol for cortical inhibiting effects in human patients. It was found to be inactive, at least by their criteria.

Cholestenone

Recently Frederickson *et al.*[34] reported that feeding Δ-4-cholestenone inhibits adrenocortical steroid secretion in the rat. Cholestenone is known to interfere with the synthesis of cholesterol (Tomkins *et al.*[35]) and it appears that this is an important link in the chain of events to corticoid synthesis. The toxicity of cholestenone is such that it will probably be of little use in man.

Streptomycin

It has been reported by Fono,[36] Boquien *et al.*[37] and Barnard[38] that prolonged treatment with streptomycin has in some cases given rise to symptoms resembling either Cushing's or the adrenogenital syndrome. Mosonyi *et al.*[39] investigated the effect of streptomycin in the adrenal response to ACTH in rabbits. They found that the expected rise in 17-ketosteroids was either augmented or suppressed, but the expected increase in glucocorticoids was consistently inhibited. Their explanation of the mechanisms involved suggests that streptomycin interferes with adrenocortical enzyme functions but this is still somewhat equivocal.

DDD and Amphenone

Two other chemical inhibitors of adrenal cortical function which have received a great deal of attention during the past decade are the insecticide, DDD, and Amphenone B.

Adrenal cortical atrophy in the dog following DDD treatment was first reported by Nelson and Woodward in 1947.[40] Nichols and Green[41] described in further detail the morphologic and functional changes resulting after 3 weeks' treatment with DDD. These investigators found the inner zone of the adrenal cortex to be atrophic while the outer glomerulosa zone was not affected by DDD; they also found that the adrenal cortex did not respond to ACTH during DDD treatment. Kuchmeister *et al.*[42] studied

the urinary excretion of 17-ketosteroids and total reducing corticoids in dogs treated with DDD and found the 17-ketosteroids to be significantly reduced, and also a suggestive reduction in other corticoids.

Subsequently, measurement of plasma 17-hydroxycorticoids in dogs by Cobey *et al.*[43,44] showed unequivocally that DDD and some of its derivatives suppressed the production of adrenal corticoids.

Further studies on this compound by Nichols,[40] Brown *et al.*[46] and Cueto and Brown [47,48] indicate that the active moiety is not pure DDD, as it had been known up to this time. *Ortho-para'*-DDD, which occurs in small quantities as a contaminant of commercial DDD, has been reported to be the active agent and may also be active in man. There is, however, still no recorded evidence that DDD or its co-geners block the secretion of aldosterone.

The other compound of interest, Amphenone B, was synthesized by Allen and Corwin[49] in 1950. This compound has been shown to exhibit an amazing gamut of biological activities. In the rat it caused: (1) adrenal hypertrophy and cholesterol deposition; (2) liver hypertrophy; (3) thyroid hypertrophy and functional inhibition; (4) anaesthetic effects; (5) acute diuresis and natriuresis; (6) thymic involution; (7) hypothermia: in the rabbit it caused progestational changes in the uterus.[50–52] Through the work of various investigators the question as to whether the hypertrophy of the adrenal cortex represents inhibition or stimulation in the rat appears to be settled. The studies of Heming *et al.*[53] and Hogness *et al.*[54] in the rat suggested that Amphenone administration increased the capacity for corticoid secretion. Vogt[55] and Holzbauer and Vogt[56] find that an initial injection of Amphenone may suppress corticoid secretion in the rat but chronic treatment does not suppress it. It has also been observed by Marks *et al.*[57] that Amphenone does not suppress cortical function in the hamster. However, it has been reported that corticoid secretion by rat adrenal slices *in vitro*[58] and by intact perfused calf adrenals[59] is clearly suppressed. The work of Hertz[60] and Nelson and Hume[61] has unequivocally established that Amphenone inhibits corticoid secretion in the dog and its corticoid inhibiting properties have also been demonstrated in man by various investigators.[62–73] Aldosterone secretion is also inhibited by Amphenone.[74,75]

The many side-effects and the toxicity of DDD and Amphenone have stimulated an intensive search for better adrenal cortical inhibitors. A recent report by Kagawa[76] indicated that 3-(3-oxo-17β-hydroxy-4-androsten-17α-yl)-propionic acid γ-lactone and its 19-nor analogue antagonize the sodium-retaining properties of desoxycorticosterone and aldosterone in the rat. This is perhaps a more specific type of inhibition than that exhibited by the various other cortical inhibitors previously described. These investigators propose that the antagonism of sodium retention is due to competitive inhibition at the re-absorptive site in the kidney tubule. Liddle[77] first reported these compounds to be active natriuretic agents in man. Several investigators have since observed that these compounds will cause sodium excretion in primary or secondary aldosteronism.[78–83] Conn[78] has reported evidence for a direct action of these compounds which is independent of their effects as competitive inhibitors of aldosterone. In general, however, it was observed that during treatment high levels of urinary aldosterone were increased further. This would tend to confirm Kagawa's hypothesis that these compounds act at the renal tubular level rather than by inhibiting the secretion of aldosterone.

Experimental Studies on New Amphenone Analogues

In our own laboratories it was decided to undertake a chemical-biological study of compounds related to Amphenone. The basic problem was to develop compounds which showed fewer of the side-effects and less toxicity than Amphenone, yet which still had the ability to suppress adrenal cortical secretion.

In this study we were faced with the problem of assaying for adrenal inhibiting properties of the compounds and for the presence or absence of side-effects and toxicity. An attempt was made at first to do all this simultaneously, and a test was developed using Amphenone as our basic standard.

Fasted male rats were simultaneously given a mild load of 0·2 per cent sodium chloride solution and a dose of Amphenone. Spontaneous urine excretion was followed for 3 h and sodium and potassium excretion during this time was measured. The rats were then continued on Amphenone treatment for 5 days, the

Table I. Amphenone effects in the rat.

Treatment	No. rats	Adrenal*	Thymus*	Testis*	Seminal vesicle*	Kidney*	Liver*	Thyroid*	Body temp† F	Na excretion† meq × 100
Controls	8	17·9	162·7	1055·5	144·8	879·0	4,930	7·25	98·0	20·9
Amphenone 20 mg/100 g × 5 da.	12	27·2	119·1	1077·0	102·7	820·0	6,117	13·1	94·4	37·9
		P = < 0·1%	P = < 0·1%		P = < 1·0%		P = < 0·1%	P = < 0·1%	P = < 1·0%	P = < 1·0%

* mg/100 g. † values obtained during standard diuretic test 3 h after initial dose.

diuresis test was repeated, and the following day the rats were autopsied and organ weights measured. It was felt that diuresis and natriuresis might be a measure of adrenal cortical inhibition, more specifically aldosterone inhibition; hypothermia, anaesthesia and loss of body weight, if present, an expression of toxicity; and of course the changes in organ weights characteristic of Amphenone were classed as undesirable side-effects. It had to be proved beyond a doubt that the natriuretic and diuretic effects in the rat correlated with cortical inhibition.

To do this, an acute test measuring the effect of these compounds on cortical secretion in dogs was developed. This was based on the technique described by Nelson and Hume[84] and by this method the complete blood flow through the adrenal gland could be collected for periods of time at any interval desired. Between collection periods the adrenal blood flow passed normally into the circulatory system. The 17-hydroxysteroid content of the collected blood samples was determined before and after Amphenone or other test compounds by the method of Silber and Busch.[85]

Table I shows the effects of Amphenone in the rat. Amphenone causes adrenal hypertrophy, thyroid hypertrophy, natriuresis and hypothermia.

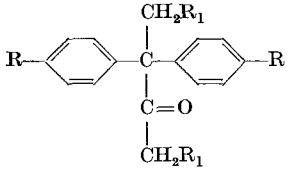
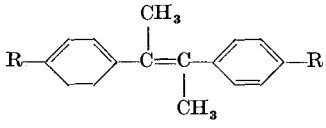
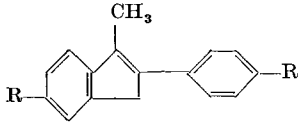
Evidence that Amphenone is also an adrenal inhibitor in the dog is illustrated by Fig. 1. Following intravenous administration, Amphenone causes an immediate drop in corticoid secretion followed by gradual recovery over a 2 h period. A dose-response effect is suggested.

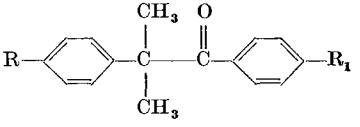
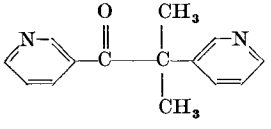
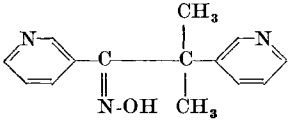
Table II shows the qualitative effects of several Amphenone analogues tested in the dog and rat, and also the ability to inhibit corticoid production by rat adrenal slices *in vitro*.

In the diphenylbutanones, the group which includes our standard, Amphenone, the various substitutions indicated have either completely destroyed or, in the case of compound (IV), decreased the ability to suppress adrenocortical function in the dog. The rat tests show that the various changes in chemical structure can abolish some of the Amphenone-like effects in that species.

The stilbenes which, under certain conditions described in the preceding paper,[86] can be obtained from the synthesis of Amphenone, show some Amphenone-like effects in the rat. Generally

Table II. The biological activity of Amphenone analogues. Rat and dog tests show activity of analogues relative to those indicated for compound (I) (Amphenone B).

Compound		R	R_1	Rat tests*		Corticoid inhibition†	
						Dog *in vivo*	rat *in vitro*
	I	NH_2	H	1. 2.	3. 4.	++	+
	II	$N(CH_3)_3^+$	H	1. 0 2. <	3. 0 4. <	0	0
	III	H	H	1. 0 2. 0	3. 0 4. <	0	0
	IV	NH_2	CH_3	1. > 2. <	3. = 4. 0	+	+
Diphenylbutanones							
	V	NH_2	—	1. = 2. 0	3. = 4. >	0	0
	VI	$N(CH_3)_2$	—	1. < 2. <	3. 0 4. =	0	0
	VII	$N(CH_3)_3^+$	—	1. < 2. 0	3. 0 4. >	0	0
Stilbenes							
	VIII	NH_2	—	1. 0 2. 0	3. 0 4. >	+	+
	IX	$N(CH_3)_2$	—	1. < 2. 0	3. 0 4. =	++	0
	X	$N(CH_3)_2$	—	1. < 2. 0	3. 0 4. =	+	0
Indenes; X = Indane							

	XI	NH_2	NH_2	1. 0 2. 0	3. = 4. =	+	
	XII	NH_2	H	1. 0 2. 0	3. < 4. 0	0‡	
	XIII	H	NH_2	1. 0 2. <	3. < 4. 0	+	
Desoxybenzoins	XIV	H	H	1. 0 2. 0	3. 0 4. =	+	0
	XV			1. 0 2. 0	3. 0 4. 0	+++	+
Pyridine (Su–4885)							
	XVI			1. 0 2. 0	3. 0 4. 0	+++	+
Pyridine							

* Key: 1. Adrenal hypertrophy 3. Hypothermia
2. Thyroid hypertrophy 4. Natriuresis

† *In vivo:* dog adrenal vein cannulation.
In vitro: rat adrenal slices.

‡ Activity of short duration.

the natriuretic effect is exaggerated. As adrenal cortical inhibitors in the dog, these compounds showed no significant effects.

In the indene series three compounds were found which showed adrenal cortical inhibition in the dog. Compound (IX), the dimethochloride, possessed activity comparable to Amphenone. In this series the rat assays indicate that thyroid hypertrophy and hypothermia have been elimiated in all the compounds and in

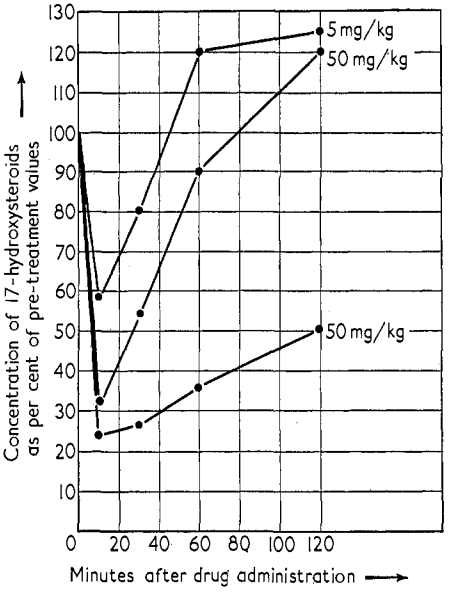


Fig. 1. Effect of various intravenous doses of Amphenone B on the concentration of 17-hydroxysteroids in blood plasma of adrenal origin

the case of compound (VIII) the only Amphenone-like effect that remains is natriuresis.

The desoxybenzoins are interesting in that the structure of compound (XI) was originally proposed to be the structure of Amphenone B. The corrected formula, a diphenylbutanone as shown for compound (I), was established in 1957 by Bencze and Allen[87] and Korman and Olson.[88] Compound (XI) still shows the ability to suppress corticoid output in the dog but no longer causes the adrenal and thyroid hypertrophy noted with Amphenone. In this series it appears that only one amino group is

necessary for the suppression of corticoid output, but the position of this group is important.

Studying the rat and dog data for these different classes of compounds discloses that in some cases it is possible to eliminate various Amphenone-like effects in the rat and still retain some cortical inhibiting properties. It is also evident that there is no

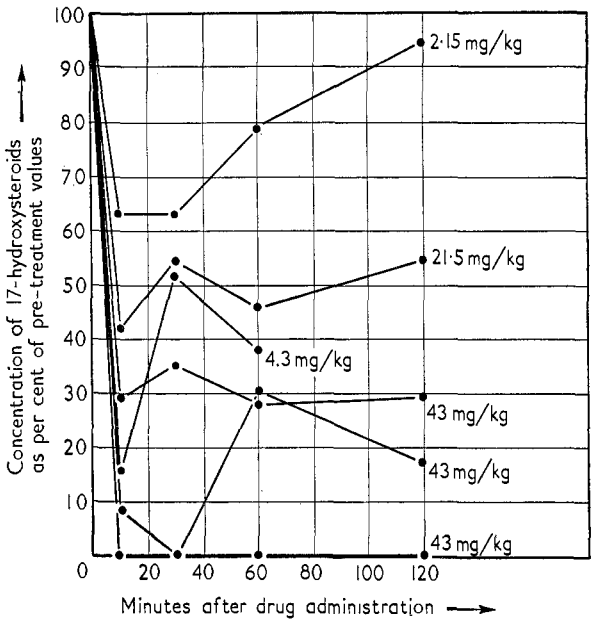


Fig. 2. Effect of various intravenous doses of Su–4885 on the concentration of 17-hydroxysteroids in blood plasma of adrenal origin

one effect or combination of effects in the rat which reliably predict cortical inhibiting activity in the dog. This is more graphically illustrated by the last two compounds, which are two of a group of pyridines described much more extensively by Bencze and Allen.[86] These compounds were essentially inactive in the rat, yet were much more potent than Amphenone as adrenal inhibitors in the dog (Chart *et al.*[57]).

Fig. 2 shows the effects of intravenous doses of compound (XV) (SU–4885) on secretion of Silber–Porter chromogens. In two of

three dogs the secretion was reduced to unmeasurable levels. This response is greater both in magnitude and duration than that observed after Amphenone. Smaller doses suggest a dose-response effect.

Initial clinical results indicated that Su-4885 was acting in man in the same manner as had been observed in the dog. Intravenous administration of Su-4885 was followed by an immediate drop in corticoid secretion as reported by Liddle *et al.*[89]

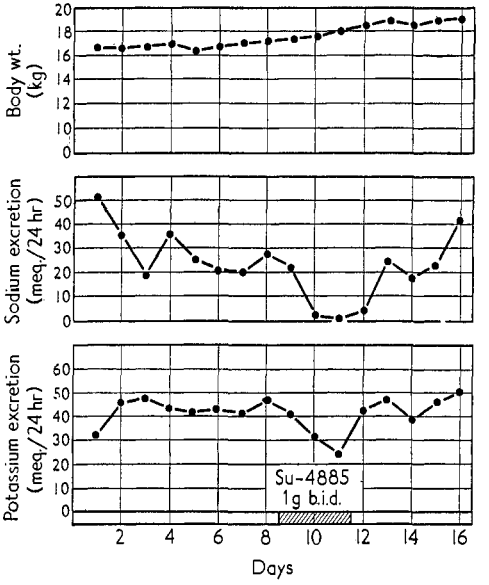


Fig. 3. Effect of Su-4885 in a dog with experimental ascites

Oral effectiveness was assumed from the results obtained after direct injection into the stomach of the anaesthetized dog, and also from the measurement of corticoid secretion by dogs on chronic toxicity tests. Following intragastric administration, the onset of effect was somewhat delayed, but the corticoid suppression followed the same pattern of results as obtained by intravenous administration. In dogs on chronic toxicity, corticoid secretion was measured 2 h after the animals had received their last oral dose of Su-4885, 200 mg/kg. Corticoid output was approximately one-third the average level shown by 86 control

dogs (Chart *et al.*[57]). Curiously enough, in toxicity studies doses up to 200 mg b.i.d. for 4 weeks did not induce adrenal insufficiency. The effects of Su-4885 were also tested in a dog with

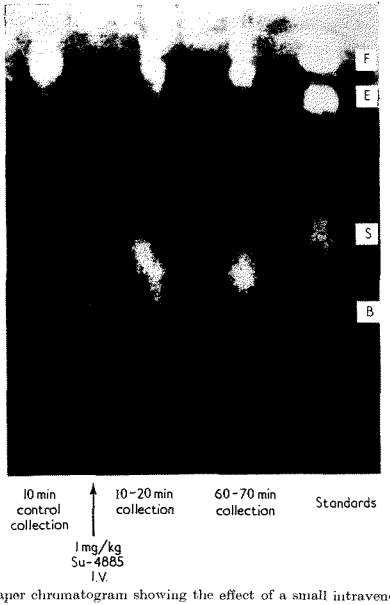


Fig. 4. Paper chromatogram showing the effect of a small intravenous dose of Su-4885 on adrenal corticoid secretion in the dog. Standard: F = hydrocortisone; E = cortisone; S = Reichstein's S; B = corticosterone

experimental ascites. In this condition one finds sodium and water retention with the accumulation of ascitic fluid, and it has also been shown that abnormally high levels of aldosterone are secreted (Davis *et al.*[90]). Su-4885 was given in an attempt to

produce natriuresis *via* an inhibition of the adrenal cortex. The data in Fig. 3 show, instead of a loss of sodium, a more exaggerated retention and weight gain.

This behaviour suggested that the immediate effects of Su–4885 on adrenal function were being counteracted by some subsequent compensatory activity, the nature of which was not clear.

At this point clarification was provided by Liddle *et al.*[89] and Jenkins and co-workers.[91] Liddle found that following oral administration or prolonged intravenous administration of the compound to man there was a marked increase in the plasma and urinary 17-hydroxycorticoids and also urinary 17-ketosteroids. Jenkins found that a large dose of Su–4885 in the dog suppressed 17-hydroxycorticoid production while with a small dose a rise was observed. Both groups observed that cortisol and corticosterone secretion were suppressed with either large or small doses, but a marked shift to the production of 11-deoxycortisol (compound S) and desoxycorticosterone (DOC) also occurred. This phenomenon adequately explains why no symptoms of adrenal insufficiency were noted in chronic toxicity studies, and why increased retention of sodium occurred in the dog with ascites.

This type of effect is illustrated in Fig. 4, which shows the chromatographic analysis of adrenal vein blood of a dog which had received 1 mg/kg of Su–4885. With this small dose we see a reduction in cortisol secretion and a marked rise in the secretion of compound S. This would indicate that although high doses can block total steroid production, the 11-hydroxylation mechanism which is the last step in the synthesis of cortisol from compound S appears to be more sensitive. Removal of the strongest natural inhibitor of ACTH production, i.e. cortisol, results in an increase in ACTH production and a consequent rise in 11-desoxycorticoid, production of which has not been blocked.

Biochemical Studies

During the development of test procedures for a study of the inhibition of adrenocorticosteroid synthesis, some *in vitro* systems were investigated. It had been shown by Rosenfeld and Bascom[92] that Amphenone was able to block the production of steroids by the perfused calf adrenal. We therefore considered the possibility

of incubating rat adrenal slices in the presence of ACTH and some of the drugs under study in a manner similar to that described by Saffron, *et al.*[93] The results of such studies are included in Table II. Only three compounds, (IX), (X) and (XIV), showing inhibition *in vivo*, failed to inhibit the production of steroids *in*

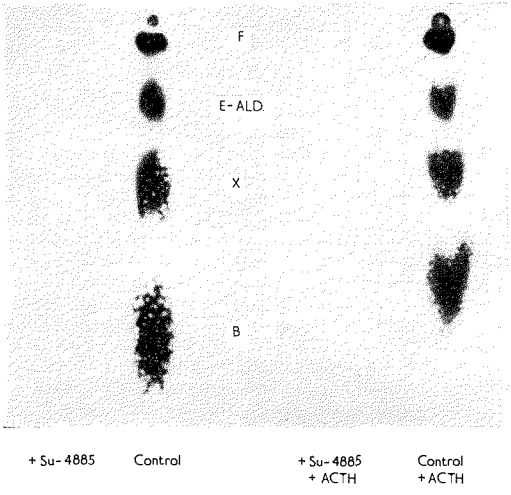


Fig. 5. Radioautograph of a paper chromatogram showing the effects of Su-4885 and ACTH on corticoid production by rat adrenals incubated with progesterone-^{14}C

vitro and two of these were compounds containing the dimethylamino group. It was interesting that these compounds produced their maximum inhibition *in vivo* somewhat later than occurred with the other compounds tested.

As a result of the growing interest in Su-4885 an examination of the manner in which it blocked steroid production by the

adrenal gland was started. In Fig. 5 may be found a radioautograph of a paper chromatogram of the steroids produced by rat adrenals incubated with progesterone-^{14}C in the presence and absence of ACTH and Su-4885. The four radioactive steroid spots fall in the areas occupied by cortisol, E-Aldosterone, X and B as described by Heard *et al.*[94] In these experiments Su-4885 was capable of markedly inhibiting the many steps involved in the production of these steroids from progesterone. It is interesting that the adrenals were able to produce approximately the same quantities of steroids from progesterone whether ACTH was present or not. A similar observation had been made by Stone and Hechter[95] in their studies with perfused calf adrenals.

Since the rat, for other reasons, did not prove to be the best animal for the study of adrenal inhibition, other species were examined. The guinea-pig adrenal offered many advantages in that it consists mostly of cortical tissue and secretes mainly cortisol. It was important, however, to determine if the guinea-pig adrenal would also respond to ACTH *in vitro*. Preliminary work found that this was indeed the case and that the response was relatively linear with time. No effort was made to pre-incubate these adrenals in order to eliminate endogenous stimulation of steroid production in spite of the fact that some synthesis occurred in the absence of ACTH. Analysis of the glands as well as the medium demonstrated that the presence of steroid in the medium was a result of net synthesis and not just secretion.

An initial study to compare Su-4885 with the reference compound, Amphenone, showed, as reported in Table III, that at a dose where Su-4885 inhibited steroidogenesis by guinea-pig adrenals quite markedly, Amphenone was completely ineffective. This failure on the part of Amphenone was evident even at higher concentrations. Subsequent studies with the guinea-pig *in vivo* failed to elicit any gross effects of Amphenone. That some species specificity existed with reference to the action of Amphenone was supported by the recent observation of Marks *et al.*[57] In their studies the hamster failed to show any effects of this drug. The *in vitro* studies indicate that Su-4885 may have a broader scope of effectiveness, in that it was capable of suppressing adrenocorticosteroid synthesis in a species not affected by Amphenone.

These initial results prompted further investigation of the mode

of action of Su–4885. Guinea-pig adrenal quarters were incubated for 2 to 3 h at 37° C in Krebs'-bicarbonate buffer containing glucose and acetate-1-^{14}C. The medium was then poured off, the slices washed with buffer, and the wash combined with the medium. The solution was then extracted with methylene chloride; the methylene chloride extract was evaporated to dryness and two-thirds of the residue chromatographed with 40 μg

Table III. Effect of Su–4885 and Amphenone B on the production of Silber–Porter positive steroids by guinea-pig adrenal slices *in vitro*.

Flask No.	Treatment*	Adrenal weight, mg	μg of Steroids as hydrocortisone	
			Secreted	in adrenal
1	Amphenone B†	89·0	3·6	1·7
1A	Control	68·2	3·4	1·4
2	Amphenone B	85·0	3·9	1·4
2A	Control	91·6	3·5	1·9
3	Amphenone B	92·6	4·1	1·7
3A	Control	73·0	3·8	1·6
4	Su–4885‡	88·0	0·9	
4A	Control	84·0	3·1	
5	Su–4885	70·0	0·9	
5A	Control	70·0	5·0	
6	Su–4885	110·0	0·7	
6A	Control	112·0	7·0	
7	Su–4885	70·0	0·9	
7A	Control	67·0	2·9	

* All flasks contained 1 i.u. of Armour lyophilized ACTH in 2 ml of Krebs–Ringer phosphate buffer.
† Concentration of Amphenone B was $9{\cdot}2 \times 10^{-4}$ M or approximately 600 μg/g of adrenal.
‡ Concentration of Su–4885 was $3{\cdot}8 \times 10^{-4}$ M or approximately 200 μg/g of adrenal.

of unlabelled cortisol as a carrier. The remaining one-third was analysed for 17-hydroxysteroids by a modification of the method of Silber and Busch.[85] The cortisol spot on the paper chromatogram was located with short-wave ultraviolet light, eluted with methanol and the ^{14}C counted with a Tricarb liquid scintillation spectrometer. The results were corrected for recoveries from the paper. The adrenals were homogenized twice with methanol, the solvent evaporated, the residue taken up in acetone: alcohol (1 : 1) and digitonin added to precipitate the free cholesterol. It

was found that these methanol extracts consisted almost exclusively of free cholesterol and that it was this portion of the total cholesterol which contained most of the counts. In order to remove and determine the total cholesterol, acetone extracts were prepared.

Preliminary studies with acetate-1-^{14}C showed that Su–4885 not only depresses the production of cortisol by the adrenal quarters but also reduces the incorporation of ^{14}C into cholesterol. Surprisingly, the presence of ACTH alone resulted in a reduced incorporation of ^{14}C into cholesterol which was reduced further by the addition of the drug.

In the study reported in Table IV, a non-incubated control was run to determine the amount of cortisol present in the gland at the beginning of the incubation period. The analysis was altered in that the total steroid of buffer and gland was analysed. It is seen that when this is done and the proper adjustment made for the steroid initially present, the specific activities of newly synthesized cortisol are roughly the same under all conditions despite the differences in the amounts of steroid produced. Since the specific activities of the cholesterol varied and were below that of the cortisol it would appear that under the conditions of the incubation the adrenal cortex prefers to synthesize cortisol without having to proceed through cholesterol. The non-obligatory role of cholesterol in the biosynthesis of adrenal steroids had been suggested earlier with perfused bovine adrenals[94] and cell-free hog adrenal preparations.[97] In order to assure that the lack of agreement of the specific activities was not a temporal artefact, the time-course of labelling of each fraction was determined and found to be linear with time. For the present, at least, this aspect of adrenal steroid synthesis must remain unsolved.

Also recorded in Table IV are results showing that while the incorporation of ^{14}C into cholesterol was depressed, that into fatty acids was stimulated and that into CO_2 was affected little, if any, by the addition of Su–4885 and ACTH. As to the effect of ACTH alone, it is observed that the incorporation of ^{14}C into fatty acids and cholesterol is depressed while that into CO_2 is unaffected. This reduced incorporation into fatty acids agrees with the observation of Hokin *et al.*[98] who reported a lowered ^{32}P content in phospholipids in the presence of ACTH. These effects of Su–4885

Table IV. Effect of Su-4885 on the incorporation of acetate-1-^{14}C into hydrocortisone and cholesterol by guinea-pig adrenal slices *in vitro*.

Flask No.	Treatment	Hydrocortisone				Fatty acids cpm	Adrenal cholesterol cpm/mg	CO_2 cpm $\times 10^3$
		μg						
		total	net	cpm	cpm/μg			
1	Su-4885 + ACTH	7·5	5·1	0	0	1,620	1,350	277
1A	ACTH	17·3	14·9	240	16·1	982	2,110	233
1B	Control	11·4	9·0	—	—	1,178	2,500	216
1C	Non-incubated	2·4	0·0	—	—	—	—	—
2	Su-4885 + ACTH	8·5	2·5	38	15·2	1,602	1,735	166
2A	ACTH	19·2	13·2	173	13·1	640	1,180	143
2B	Control	12·6	6·6	90	13·7	7,302	3,720	206
2C	Non-incubated	6·0	0·0	—	—	—	—	—
3	Su-4885 + ACTH	6·6	6·6	40	6·1	396	377	87
3A	ACTH	14·8	14·8	128	8·8	132	612	80
3B	Control	7·8	7·8	62	7·0	572	1,270	68
3C	Non-incubated	0·0	0·0	—	—	—	—	—

could be repeated with demedullated adrenals indicating that the medulla exerted little or no influence on the ^{14}C incorporation.

Since glucose metabolism *via* the hexose monophosphate (HMP) shunt has been reported to play such a key role in the biosynthesis of the steroid hormones[97] it was considered important to study the effects of Su–4885 on the metabolism of glucose-1-^{14}C and glucose-6-^{14}C. In Table V we see that Su–4885 reduces the incorporation of ^{14}C from both labelled glucose species into CO_2, fatty acids, total lipids and cholesterol. The ratios of ^{14}C derived from glucose-1-^{14}C to that derived from glucose-6-^{14}C for fatty acids, total lipids and cholesterol were found to be 0·55, 0·5 and 0·33 respectively. This would imply that 45–67 per cent of the labelled glucose is oxidized *via* the HMP shunt mechanism. The importance of this route agrees with the observations of Kelly, *et al.*[99] concerning the high level of glucose-6 phosphate dehydrogenase activity found in the adrenal cortex. The addition of Su–4885 results in a non-selective inhibition of both routes of metabolism of glucose, since again the percentage traversing the HMP shunt is found to range from 58–67 per cent.

These tracer studies have constituted an attempt to explain the manner in which Su–4885 is able to inhibit selectively or completely the steroid production of the adrenal glands. A major stumbling block to an adequate interpretation of the data lies in the poor understanding we have of the manner in which ACTH effects its stimulation of steroid production. The work of Kelly, *et al.*,[99] pointing out the high glucose-6 phosphate dehydrogenase levels in the adrenal, coupled with the more recent works of Haynes and Berthet[100] and Haynes,[101] pointing to the stimulating activity of ACTH on the production of 3′,5′-AMP and thus the activation of adrenal phosphorylase, stress the importance of glucose metabolism particularly *via* the HMP shunt. This route of glucose oxidation will generate reduced triphosphopyridine nucleotide (TPNH) which is necessary for the hydroxylation reactions involved in the production of the adrenocorticosteroids. From the studies with radioactive glucose it would appear that the effect of Su–4885 on steroid biosynthesis could result from its inhibition of glucose metabolism. The point at which the inhibition occurs must remain in the realm of conjecture, although one might venture to look at some step leading to the formation of

Table V. Effect of Su–4885 on the metabolism of glucose-1-^{14}C and glucose-6-^{14}C by guinea-pig adrenals *in vitro*.

Flask No.	Treatment	Substrate	Counts/Minute/Gram of Adrenals in			cpm/mg of cholesterol
			CO_2 $\times 10^5$	Fatty acids	Total lipids $\times 10^4$	
1	Su–4885	Glucose-1-^{14}C	0·84	162	0·68	88
1a	Control		3·2	162	2·06	314
4	Su–4885	Glucose-6-^{14}C	2·0	906	1·70	198
4a	Control		2·9	1,530	4·93	1,140
2	Su–4885	Glucose-1-^{14}C	1·0	284	0·59	125
2a	Control		2·4	946	1·80	345
5	Su–4885	Glucose-6-^{14}C	1·1	760	1·82	284
5a	Control		3·5	1,025	3·97	1,270
3	Su–4885	Glucose-1-^{14}C	0·45	204	0·51	9
3a	Control		2·7	717	1·59	282
6	Su–4885	Glucose-6-^{14}C	0·91	288	0·67	290
6a	Control		2·5	1,160	2·05	805

Each flask contained two guinea-pig adrenals plus 0·21 μmoles of glucose^{14}C and 1 i.u. of ACTH/2 ml of Krebs-bicarbonate buffer. Concentration of drug when used was 400 mg/l.

glucose-6 phosphate. From the standpoint of the exogenous labelled glucose this would be at the level of the hexokinase reaction. However, we had established in preliminary experiments that steroid production by the guinea-pig adrenal in the presence of ACTH was affected little, if any, by the presence or absence of glucose in the medium. This implies that sufficient quantities of glucose-6 phosphate are generated endogenously, probably from glycogen, for the subsequent generation of TPNH *via* the glucose-6 phosphate dehydrogenase reaction. Inhibition by Su-4885 of one of the reactions leading to the production of glucose-6 phosphate from glycogen would result in decreased glucose oxidation with its subsequent reduction in levels of ATP as well as TPNH. In the presence of a decreased level of ATP, the hexokinase catalysed reaction which is responsible for the phosphorylation of exogenous glucose would be inhibited. This would help to explain the reduced metabolism of the labelled glucose supplied in the medium. The reduced levels of TPNH would, of course, result in an inhibition of many of the hydroxylation reactions involved in the synthesis of the adrenal steroids.

Certain observations made in these studies are rather difficult to explain according to present concepts of intermediary metabolism. It can be calculated that the number of μmoles of glucose oxidized to CO_2 is one-fourth that of acetate. Since it would appear, as mentioned above, that exogenous glucose probably constitutes only a small fraction of the total carbohydrate being oxidized in the presence of ACTH, it is strange that one finds essentially no change in the $^{14}CO_2$ levels from acetate upon the addition of ACTH. One should certainly expect some dilution of the labelled acetyl-Co A by unlabelled acetyl-Co A derived from endogenous carbohydrates which would be reflected in a reduced $^{14}CO_2$ production. However, this did not occur in our experiments and an examination of the literature demonstrates that this dilution effect was also absent in studies of a similar nature. The question arises as to whether the acetate pool is homogeneous or compartmentalized. Perhaps the acetyl-Co A generated from endogenous precursors does not mix completely with that derived from exogenous acetate. Freedman and Graff[102] recently demonstrated a remarkable stability of oxalacetate derived from endogenously generated pyruvate, whereas exogenous oxalacetate was very rapidly

decarboxylated. This suggestion of possible compartmentalization of co-enzymes and metabolic intermediates deserves further attention, and if found actually to exist could affect our interpretation of a great deal of isotope data.

The rise in incorporation of acetate-1-^{14}C into fatty acids in the presence of Su–4885 and ACTH appears strange in the face of a decreased incorporation into cholesterol. It would appear from the glucose data that fatty acid synthesis is not stimulated, since the ratio of ^{14}C incorporated into CO_2 to that incorporated into fatty acids and total lipids was the same with or without the drug. However, Siperstein and Fagan[103] had demonstrated that TPNH generated by glucose-6 phosphate oxidation is utilized for cholesterol synthesis in preference to that generated *via iso*citrate oxidation. In their experiments, when *iso*citrate was substituted for glucose-6 phosphate, more acetate-1-^{14}C was incorporated into fatty acids and less into cholesterol. In our experiments with Su–4885 in the presence of acetate-1-^{14}C less TPNH would be formed *via* glucose-6 phosphate oxidation and more would be generated *via iso*citrate oxidation. Therefore, in a manner analogous to that observed by Siperstein and Fagan,[103] the incorporation of ^{14}C into fatty acids increased while that into cholesterol decreased.

Effects on Carbohydrate Metabolism

As is well known, corticoids normally antagonize the effect of insulin on blood sugar and in the absence of corticoids there is an exaggerated fall in blood sugar following insulin. It was reasoned then that if Su–4885 caused inhibition of cortisol secretion, an increase in sensitivity to insulin would be observed.

Rats were treated twice a day for 2½ days with Su–4885 (a total of 5 doses) and insulin was administered 2 h after the last dose. Blood sugar* and liver glycogen† were measured 3 h after insulin. These results are shown in Fig. 6. Compared to controls, there was a marked increase in liver glycogen in the Su–4885 treated animals. The slight decrease in blood sugar is not significant.

* Blood sugar and glucose equivalent of liver glycogen measured by the method of Nelson.[107]

† Precipitation and hydrolysis of glycogen; *cf.* Chart, *et al.*[108] All liver glycogen figures reported are glucose equivalent of glycogen.

The insulin-treated animals of course show a significant decrease in blood sugar when compared to controls.

In the animals receiving both Su–4885 and insulin there is a surprising fall in blood sugar which is much greater than can be explained by any additive effects of the two compounds. The liver glycogen effect of Su–4885 is also no longer evident. The

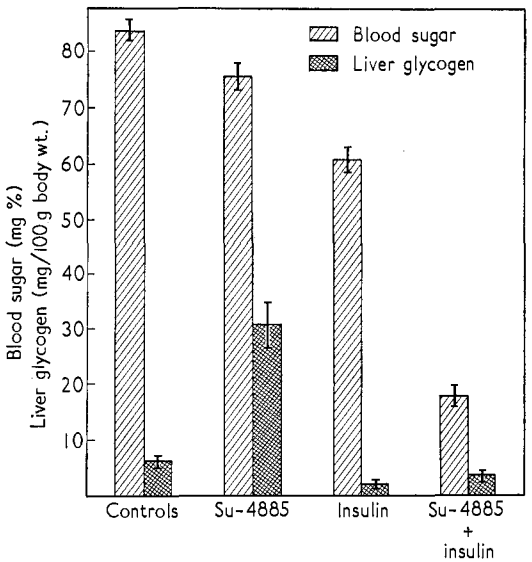


Fig. 6. Showing individual and combined effects of Su–4885 and insulin on carbohydrate metabolism in the rat. Number of rats = 12/group. Su–4885 dose = 129 mg/kg b.i.d. for 5 doses. Insulin dose = 3 U/kg given 2 h after last dose of Su–4885. Rats fasted 18 h before autopsy. Autopsy 5 h after last dose of Su–4885. (I) = standard error

increase in sensitivity to insulin in this test could possibly indicate an inhibition of adrenal cortical secretion. However, the rise in liver glycogen in the animals receiving Su–4885 alone would not be expected with corticoid inhibition.

It was also found that this response obtained if measured acutely (5 h after a single dose) or after chronic treatment with Su–4885 for periods up to $8\frac{1}{2}$ days. The $2\frac{1}{2}$ day treatment used originally was based on the premise that if a response were to be effected through adrenal inhibition it would require more than an

acute treatment. This apparently was not necessary to elicit the response, and also it would appear, as determined by 8½ days of treatment, that adaptation to the effects of Su–4885 did not occur.

To test whether or not Su–4885 possessed true hypoglycaemic properties, alloxan diabetic animals were treated for varying lengths of time, both with and without insulin.* Su–4885 exhibited no effects by itself nor did it potentiate the effect of insulin.

The marked increase in liver glycogen levels of rats treated with Su–4885 suggested that one mechanism of action might be through an inhibition of the action of glucagon.† Animals were treated with Su–4885 and 4 h later were given 0·1 mg of glucagon. Blood

Table VI. Effect of Su–4885 and glucagon on blood sugar and liver glycogen in the rat.

Treatment	Blood sugar, mg %	Liver glycogen, mg/100 g body weight
Controls	89·9	8·5
Su–4885, 129 mg/kg	122·1	51·5
Su–4885, 129 mg/kg + Glucagon, 0·1 mg	112·1	1·1
Glucagon, 0·1 mg	108·0	1·2

sugar and liver glycogen levels were determined 1 h after glucagon. The data in Table VI indicate that the effect of glucagon is not altered by pre-treatment with Su–4885; liver glycogen stores are similarly depleted in both glucagon controls and animals pre-treated with Su–4885. In all previous experiments blood sugar and liver glycogen were determined 5 h after Su–4885 administration. The time-course of events was then established by taking these measurements at hourly intervals during this 5 h period. The results in Fig. 7 show an effect on blood sugar that had been missed completely due to the timing of the original procedure.

* The help of Dr. A. A. Renzi, who conducted the alloxan diabetic tests, is gratefully acknowledged.

† Dr. Mary A. Root, Lilly Research Laboratories, Indianapolis, Indiana, kindly supplied the glucagon.

There was a marked increase in blood sugar, which reached a peak at about 2 h and then returned to control levels at 5 h. This pattern of response is very similar to that obtained with epinephrine.[104]

These effects on blood sugar and liver glycogen in the rat are quite specific for Su–4885. Amphenone, compound (XVI) (Table II) and other closely related compounds did not show this type of activity.

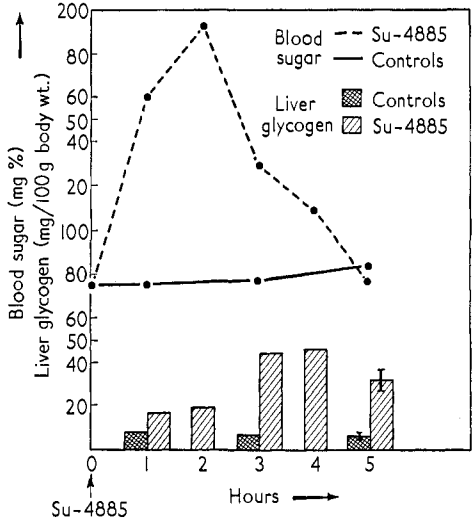


Fig. 7. Time-course response of blood sugar and liver glycogen following administration of 129 mg/kg Su–4885 in rats fasted 18 h. (I) = standard error

This sharp rise in blood sugar and the increased sensitivity to insulin, observed in previous experiments, seem somewhat paradoxical. However, a review of the first protocol shows that insulin was administered 2 h after Su–4885, when the blood sugar response was at its peak. Hypothetically, this high blood sugar level may have served as a stimulus to increase the secretion of insulin which, when added to that given by injection, gave the marked fall in blood sugar. This hypothesis may very well explain why there was no marked sensitivity to insulin in the alloxan diabetic animals.

When Su-4885 and insulin were given simultaneously and the blood sugar and liver glycogen measured 3 h later, the carbohydrate effects of Su-4885 were negated. Insulin alone showed its typical hypoglycaemic effect and Su-4885 alone showed the expected hyperglycaemia and deposition of liver glycogen.

To test whether or not these carbohydrate effects were mediated *via* the adrenal, blood sugar levels were measured in adrenalectomized rats at hourly intervals after Su-4885, and in rats which

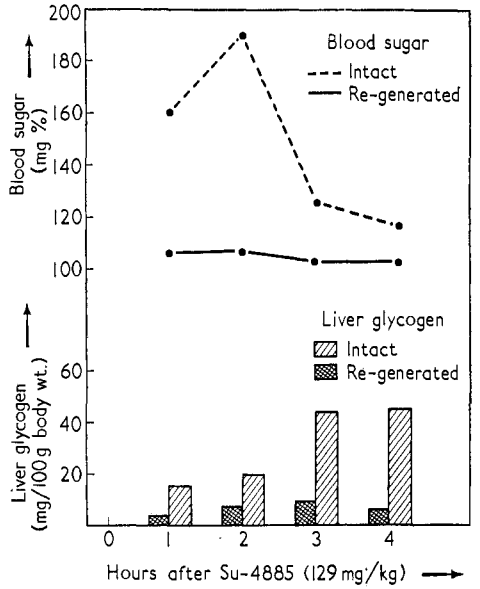


Fig. 8. Effect of Su-4885 on blood sugar and liver glycogen in rats with intact or regenerated adrenals

had their adrenals enucleated and then allowed to regenerate for 2 weeks prior to the test. Fig. 8, showing the results in the enucleate animals, indicates that the response is essentially abolished. The adrenalectomized animals did not differ from the enucleate group. There is some slight response in the enucleate animals but these results would indicate that: (1) the carbohydrate response is adrenal mediated; and (2) the carbohydrate response is dependent mainly on the adrenal medulla.

The possibility that these carbohydrate effects might be

attributed to epinephrine release was tested further. Rats were given Su–4885 and at hourly intervals thereafter groups of animals were sacrificed; blood was taken for blood sugar analysis,

Table VII. Effect of Su–4885* on blood sugar and adrenal catecholamine content in the rat.

Time, h	Blood sugar, mg %	Adrenal catecholamine content, μg/mg tissue
Control	97·5	1·19
1	214·0	1·05
2	193·6	0·53
3	137·5	0·69
4	149·7	0·63

* Su–4885, 129 mg/kg.

and the adrenals were removed for the measurement of catecholamine content.* Table VII shows the typical rise in blood sugar levels, while the adrenals concurrently show a 50 per cent reduction in catecholamine content. Catecholamine content of brain and heart showed no change.

Table VIII. Effect of Su–4885* on blood sugar, liver glycogen and adrenal blood catecholamine content in the dog.

Time, min	Adrenal vein catecholamines, μg/ml	Blood sugar, mg %	Liver glycogen, mg/100 g liver
60	0·014	94·3	33·98
5	0·091	121·5	—
15	0·181	146·6	33·0
30	0·116	151·8	35·0
60	0·125	144·5	35·4
120	0·10	146·7	32·1

* Su–4885, 50 mg/kg.

Catecholamine content of adrenal blood in the dog was also measured by our cannulation technique. A control blood sample was taken and, at various intervals after Su–4885, adrenal blood

* Catecholamines determined by condensation with ethylene diamine under conditions described by Weil-Malherbe and Bone.[109]

was analysed for blood sugar and catecholamines. The data in Table VIII show that following Su–4885 there is approximately a ten-fold increase in catecholamine concentration of adrenal vein blood and there is also an increase in blood sugar.

Summary and Conclusions. A review of surgical procedures and chemotherapeutic agents which suppress cortical function has been presented, including experimental studies with new analogues of Amphenone. These studies have demonstrated that compounds can be made which retain adrenal inhibiting properties but have fewer additional actions than Amphenone. One group in particular, which has two pyridine groups in place of the aniline groups of Amphenone, received special attention because of the reduced toxicity and greater activity of some of its members as compared with Amphenone. Su–4885 was singled out from this group for more extensive laboratory and clinical study.

Initial experiments indicated that Su–4885 was a potent inhibitor of adrenal corticoid production. In the dog and man the initial effect of Su–4885 is to inhibit the secretion of all 17-hydroxycorticoids, which then results in an outpouring of ACTH. This increased production of ACTH cannot, however, completely correct the depressed corticoid output, in that 11β-hydroxylation reactions (e.g. hydrocortisone production) remain inhibited. The cortex, therefore, responds by producing in excess the 11-desoxysteroids, compound S and DOC. These actions serve as the basis for use of the compound in the differential diagnosis of pituitary–adrenal dysfunctions.[89, 105, 106, 110]

In vitro studies suggest that this inhibitory action on steroid production might be attributed to a decrease in glucose metabolism in the adrenal cortex.

Interesting effects of Su–4885 on carbohydrate metabolism in the rat and dog have been demonstrated and appear to result from the release of catecholamines from the adrenal medulla.

The potential therapeutic value of Su–4885 is as yet somewhat questionable but is, nevertheless, actively being investigated. The chemical-biological effort which has produced this compound, however, has resulted in the development of a valuable diagnostic tool in the clinic and a technique for the further study of corticoid biosynthesis in the laboratory.

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