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GLYCYRRHIZA

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GLYCYRRHIZIN
and
DERIVATIVES

Summary

Licorice, an extract from the roots of the licorice plant, Glycyrrhiza glabra, has been manufactured and used by man for many centuries. References to licorice are found in many of man's earliest medical records, including those of the Greek physican Hippocrates. A vast number of ailments, mainly affections or inflammations of the respiratory tract, have, from time to time, been reported as curable by the extract. Even today a variety of potions and medicants contain licorice.

The licorice extract contains a compound, glycyrrhizin, which is about 50 times sweeter than sucrose. This compound is generally considered responsible for the medicinal qualities of licorice.

Little work has been done on the metabolism of glycyrrhiza and what little work that has been done is somewhat contradictory. Using tritium - labelled monoammonium glycyrrhizinate (MAG), Carlat et al. (13) determined that MAG is only slightly absorbed from the human gastrointestinal tract but is hydrolyzed to form glycyrrhetic acid* (GA), which is excreted unchanged in the feces. On the basis of experimental data, the possibility of MAG being absorbed into the blood and then excreted via the bile was discounted (13). The oral administration of labelled GA by Carlat et al. (13) produced essentially the same results. However, Parke et al. (106), following administration of tritium labelled beta-GA to rats with biliary cannulae, reported that most of the compound was absorbed and excreted in the bile before being excreted in the feces. Van Katwijk and Huis in't Veld (132) reported that analysis of the urine of two human patients given orally 2.28 and 2.5 g, respectively, of glycyrrhizic acid, daily, failed to show any of the unaltered compound, however an unidentified metabolite of the acid was present.

A number of biochemical parameters have been reported to be effected by licorice and/or glycyrrhiza. In vitro experiments by Whitehouse et al. (139) have shown that glycyrrhetic acid is a potent uncoupler of oxidative phosphorylation in rat liver mitochondria. Noda (102) reported that glycyrrhizin inhibits the enzymatic activity of proteinase and of a homogenate prepared from dog spleen. Kraus (69) found that ammoniated glycyrrhizinate decreased the ability of rats to mobilize glucose and to withstand cold temperatures, suggesting that glycyrrhizin decreases the output of ACTH. Eudokimova and Kamilou (24) found that potassium glycyrrhizinate shows a positive effect on the course of experimental atherosclerosis in rabbits by decreasing the amount of cholesterol in the blood and reducing the cholesterol-lecithinic coefficient. Hassan et al (52), Linko and Vasama (81), Finney et al. (26), Galal (33), and Card et al. (12) found that GA exhibits a marked

*Since GA is a metabolite of glycyrrhiza it will be discussed in this monograph along with the other compounds.

antidiuretic action and depresses urinary excretion of sodium and chloride. Hassan et al. (52) reported similar effects with ammoniated glycyrrhizinate and monoammonium glycyrrhizinate. Oral administration of 2 g of glycyrrhizic acid daily was found by Revers (115) to produce edema in in human subjects. Revers (115) reported that extract of licorice contains a substance having spasmolytic power and probably influencing the healing process of gastric ulcers.

In studies on rats and humans with peptic ulcers, Nishiyama (101) found that licorice exhibited a therapeutic effect while glycyrrhizin did not.

Sasano et al. (121) reported that simultaneous, intravenous administration of glycyrrhizin with dexamethasone inhibits the dexamethasone-induced atrophy of the adrenals of rats.

Asanuma (4) found that glycyrrhizin can either suppress or intensify the action of cortisone, depending upon the immediate conditions, and can suppress the inhibitory action of dexa methasone on the pituitary. The antiarthritic action of hydroxycortisone was found by Gujral et al. (47) and Elmadjiam et al. (23) to be potentiated by glycyrrhizin and monoammonium glycyrrhizinate, respectively.

To study the teratologic effects of ammonium glycyrrhizinate, the compound was given, per os, to pregnant mice, rats, hamsters, and rabbits in doses as high as 1000 mg/kg/day for periods up to 18 days (28). Detailed examinations of the fetuses and mothers indicated that ammonium glycyrrhizinate exhibited no teratologic effect nor unfavorably influenced maternal or fetal survival (28).

A table of acute toxicity is presented on page 11.

The few feeding studies on licorice and glycyrrhiza which have been reported to date have been restricted to rats and man.

For a period of 50 days, Girerd et al. (39) administered, intragastrically, 10 g of licorice/kg/day to 14 rats (Group B), gave 1 g of ammoniated glycyrrhizin/kg/day to 13 rats (Group C), and maintained 10 rats as controls (Group A). The treated animals exhibited a pronounced rise in blood pressure and a significant depression of growth, the growth depression being greater in Group B. The survival rate of the animals was 100, 77, and 36% for Groups A, B, and C, respectively (39). Increases in weight were noted for the kidneys, adrenals, and heart of the treated animals while decreases were found in the weight of the hypophyses and testes (39). Severe renal and cardiovascular lesions were found in Group B, with milder lesions being found in Group C and none in the controls (39).

Macabies et al. (86) found the daily administration, per os, of 160 mg of glycyrrhizin to 30 male Wistar rats to produce a constant, lasting, and reversible elevation of blood pressure. In a further study, Macabies et al. (85) found that the salts of glycyrrhizic acid and the isomers of glycyrrhetic acid exert a strong hypertensive action while the "deglycyrrhizinated" extracts of licorice showed only a very weak hypertensive action.

In a study by Fujimura and Okamoto (32), 10 groups of 5 rats where given di-potassium glycyrrhizinate and di-ammonium glycyrrhizinate at dietary levels of 0, 0.1, and 0.5% for a period of 90 days. The only untoward effect observed was a decrease in body weight gain for the male rats given the highest level of treatment (32).

Klosa (67) observed no untoward effects when rats were given potassium glycyrrhizinate at a level of 60 mg/kg/day for a period of 8 months.

In three feeding studies (12, 84, 94) employing human subjects, increases were found in water, sodium, and chloride retention and in potassium excretion. In these studies, Molhuysen et al. (94) used succus liquiritiae (20-45 g/day - 10 subjects), Louis and Conn (84) used ammonium glycyrrhizinate (as much as 6 g/day for 3 days or 4 g/day for 5 days - 10 subjects), and Card et al. (12) used black juice (20 or 36 g/day for 4 days - 2 subjects). Louis and Conn (84) also noted a significant decrease in 17-ketosteriods, indicating inhibition of the pituitary - adrenal system. Molhuysen et al. (94) and Card et al. (12) noted increases in blood pressure and Molhuysen et al. (94) found the hemoglobin and total serum protein levels to be decreased.

In a case study reported by Chamberlain (14), a 53-year-old man consumed 700 g of licorice candy within a period of 9 days and as a result manifested high blood pressure and hypokalemia. A 2-gram sodium diet and bed rest eradicated the symptoms in 4 days (14).

Harders and Rausch-Strooman (51) reported that a 48-year-old man consumed a 35 g licorice bar every other day for a period of approximately 6 months. The resultant high blood pressure was corrected, within 14 days, by a salt-free diet, bed rest, and mild laxative and sedation therapy (51).

Rouques (117) reported that 5 cases of licorice-induced intoxication, characterized by hypertension and polydipsia, were rectified by elimination of the glycyrrhizin.

Cramptom (17) reported a case of a 19-year-old woman who suffered from edema as a result of licorice consumption. Abstinence from licorice rectified the edema (17).

Two cases of glycyrrhizin intoxication reported by Potton et al. (113) manifested constant arterial hypertension, sometimes severe neuromuscular symptoms with occassional tetanic manifestations and a biological syndrome characterized by metabolic alkalosis with severe hypokalomia and hypernatremia. Intoxication developed only when 1 g/day or more was ingested and disappeared completely within 20 days of cessation of ingestion (113).

Chemical Information

Glycyrrhizin

I. Nomenclature

A. Common Names

1. Glycyrrhizin
2. Glycyrrhizic acid
3. Glycyrrhizinic acid
4. Glycyrrhetinic acid glycoside

B. Chemical Name

1. Glycyrrhizin

C. Trade Name

(none)

D. Chemical Abstracts Registry No.

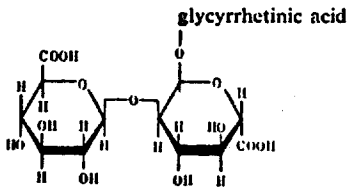
977004311

II. Empirical formula

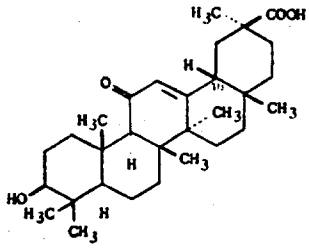
$C_{42}H_{63}O_6$

III. Structural Formula

Glycyrrhizin is a beta,beta'-glucuronido glucuronide of glycyrrhetinic acid.



glycyrrhizin



glycyrrhetinic acid

An alternative name for glycyrrhetinic acid is 3 beta-hydroxy-11-oxoolean-12-en-30-oic acid.

IV. Molecular Weight

822.92

VI. Description

A. General characteristics

Intensely sweet taste.

B. Physical properties

Freely soluble in hot water, alcohol; practically insoluble in ether.

VII. Analytical Methods

Glycyrrhizin is used in many commercial products. Licorice extract may be purified by adding alcohol to an aqueous solution until a 63% alcohol concentration is attained. The starch and gums are precipitated and filtered off. The addition of sulfuric acid precipitates the glycyrrhizin, which is further purified by washing in warm 95% alcohol and filtration. After evaporation and drying, the weight as glycyrrhizin is determined. There is some question as to the quantitation of the precipitation (100).

After the initial precipitation of glycrrhizin by sulfuric acid, the washed precipitate may be redissolved in alcohol and titrated with standard alkali. However, other acid constituents of the licorice extract may interfere (100).

After the licorice solution has been purified, glycyrrhizin can be precipitated by adding copper sulfate. The copper glycyrrhizinate may be dried and weighed directly, or ignited and weighed as the copper oxide (100).

One colorimetric determination of glycyrrhizin is based upon hydrolysis of the glycoside and reaction of the liberated hexuronic acid with naphtoresorcinol in an acidified medium. The violet reaction product is extracted with benzene (100).

A more specific determination of glycyrrhizin depends on the liberation of the glycyrrhetic acid component. After alcohol extraction of the sample and precipitation of glycyrrhizin with saturated neutral lead acetate solution, glycyrrhetic acid is liberated by refluxing with dilute mineral acid. After extraction of the glycyrrhetic acid with chloroform, evaporation, and dissolution in sulfuric acid, a bluish violet color is developed upon reaction with vanillin solution (100).

Glycyrrhizin from block licorice may be assayed by a polarographic method. The sample is dissolved in 1:1 dioxane-water mixture, followed by hydrolysis with dilute acid. The resulting glycyrrhetic acid is chloroform extracted, evaporated, and the residue brought into solution in a diluted alcoholic, acetate-buffered medium. This is then polarographed, dependent on the electroactivity of the unsaturated keto group of glycyrrhetic acid (100).

Larry et al. developed a gas-liquid chromatographic method for the quantitation of ammonium glycrrhizinate as the aglycone, glycyrrhetic acid. The acid hydrolysate is extracted with chloroform, dried, and silylated. This product and an internal standard are then chromatographed. Recoveries from both water and sodas containing caramel averaged 87% (78).

VIII. Occurrence

Plant

Glycyrrhizin comes from the dried rhizome and roots of *Glycyrrhiza glabra L.*, variety typica (Spanish licorice), or of *G. glabra* L., variety *glandulifera* (Russian licorice), or of other varietys of G. glabra yielding a yellow and sweet wood, Leguminosae.

Glycyrrhizin may also be extracted from the roots of Abrus precatorius, Periandra dulcis, Periandra mediteranea, and from the bark of the trees, Lucuma glycyphylla, Achras sapota, and Sideroxylon richardii.

Ammonium Glycyrrhizinate

I. Nomenclature

A. Common Name

Ammonium Glycyrrhizinate

B. Chemical Name

Ammonium glycyrrhizinate

C. Trade Name

(none)

D. Chemical Abstracts Registry No.

001407030

II. Empirical formula

$C_{42}H_{61}O_{16}NH_4 \cdot 5H_2O$

III. Structural Formula

Ammonium glycyrrhizinate is the ammonium salt of glycyrrhizin. (See glycyrrhizin).

IV. Molecular Weight

929.92

V. Specifications

No information available.

VI. Description

A. General characteristics

Ammonium glycyrrhizinate may be recovered from 75% ethanol in a needle form.

B. Physical Properties

Ammonium glycyrrhizinate decomposes between 212-217° C, has an absorption maximum at 248 nm., and is soluble in ammonia water and glacial acetic acid.

C. Stability

No information available.

VII. Analytical Methods

(See glycyrrhizin)

VIII. Occurrence

Plant ammonium glycyrrhizin comes from the dried rhizome and roots of Glycyrrhiza glabra L., variety lypica (Spanish licorice), or of G. glabra L., variety glandulifera (Russian licorice), or of other varietys of G. glabra yielding a yellow and sweet wood, Legumirosue.

Ammonium glycyrrhizin may also be extracted from the roots of Abrus precatorius, Periandra dulcis, Periandra mediteranea, and from the bark of the trees, Lucuma glycyphylla, Achras sapota, and Sideroxylon richardii.

Licorice

I. Nomenclature

A. Common Names

1. Licorice
2. Liquorice
3. Glycyrrhiza
4. Sweet root
5. Radix liquiritiae (drug form)
6. Succus liquiritae (crudem)
7. Extractum liquiritiae (venale)
8. Extractum glycyrrhizae (crudem or siccum)
9. Block licorice

B. Chemical Name

Glycyrrhiza

C. Trade Name

Glycine

D. Chemical Abstracts Registry No.

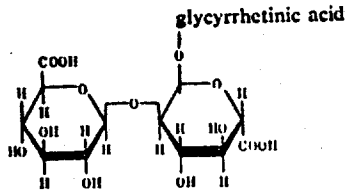
977004311

II. Empirical Formula

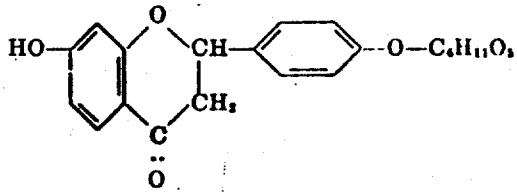
Not applicable

III. Structural Formula

The term licorice applies to the dried rhizome and roots of the Glycyrrhiza glabra L.; hence, licorice is a mixture of several constituents. It contains 6-14% glycyrrhizin, a beta, beta'-glucuronido-glucuronide of glycyrrhetinic acid. A second glycoside, 7 - hydroxy-4'-glucosidoxy-flavenone, has been found present in licorice extract (100).



glycyrrhizin



7-hydroxy-4'-glucosidoxy-flavenure

Also, an ether-insoluble resin consisting of glucuronic acid and a hemolytic sapogenin is present. Mono-, di, and poly saccharides have been found, especially D-glucose, saccharose, sucrose, starch, and possibly pectin; the starch content varies around 22%. Further constituents of the licorice root are fat around 1%, L-asparagine about 3%; and bitter compounds to a total of 10%. Minute amounts of essential oil, tannin, and methylsalicylate occur (100).

IV. Molecular weight

(not applicable)

V. Specifications

No information available.

VI. Description

A. General Characteristics

Licorice extract is hard, brittle, and has a brownish-black color.

B. Physical Properties

(not applicable)

C. Stability

VII. Analytical Methods

See glycyrrhizin

VIII. Occurrence

Plant licorice comes from the dried rhizome and roots of Glycyrrhiza glabra L., variety lypica (Spanish licorice), or of G. glabra L., variety glandulifera (Russian licorice), or of other varietys of G. glabra yielding a yellow and sweet wood, Legumirosue.

Licorice may also be extracted from the roots of Abrus precatorius, Periandra dulcis, Periandra mediteranea, and from the bark of the trees, Lucuma glycyphylla, Achras sapota, and Sideroxylon richardii.

Biological Data

I. Acute Toxicity

Substance*	Animal	Sex & No.	Route	Dosage (mg/kg)	Measurement	Ref.
CAG	Mice	-	i.p.	1050	LD_{50}	31
CAG	Mice	-	p.o.	12700	LD_{50}	31
CPG	Mice	-	i.p.	1260	LD_{50}	31
CPG	Mice	-	p.o.	12400	LD_{50}	31
MAG	Mice	-	i.p.	1070	LD_{50}	31
DPG	Mice	-	i.p.	1400	LD_{50}	32
DPG	Mice	-	p.o.	8100	LD_{50}	32
DAG	Mice	-	i.p.	1250	LD_{50}	32
DAG	Mice	-	p.o.	9600	LD_{50}	32
GA	Mice	-	i.p.	308	LD_{50}	26
FM100	Mice	10 M	i.v.	251	LD_{50}	129
FM100	Mice	60 M	i.p.	641	LD_{50}	129
PG	Mice	-	i.v.	412	LD_{50}	67
PG	Mice	-	i.m.	695	LD_{50}	67
PG	Mice	-	s.c.	697	LD_{50}	67
PG	Mice	-	p.o.	1220	LD_{50}	67

* Abbreviations used: CAG - crude ammonium glycyrrhizinate, CPG - crude potassium glycyrrhizinate, MAG - monoammonium glycyrrhizinate, DPG - di-potassium glycyrrhizinate, DAG - di-ammonium glycyrrhizinate, GA - glycyrrhetic acid, F_m 100 - an extract of licorice, PG - potassium glycyrrhizinate.

Using groups of 6 mice weighing from 14 to 17 g, Fujimura determined the LD_{50}'s of crude ammonium glycyrrhizinate (CAG), crude potassium glycyrrhizinate (CPG), and mono-ammonium glycyrrhizinate (MAG) administered both orally and intraperitoneally. The animals were observed for general condition and mortality for a period of 48 hours following administration of the test materials. The results are presented below (31).

Sample	LD_{50} (mg/kg)	
	i.p.	p.o.
CAG	1050	12700
CPG	1260	12400
MAG	1070	-

Aqueous solutions of di-potassium glycyrrhizinate (DPG) and di-ammonium glycyrrhizinate (DAG) were administered intraperitoneally or orally at various dosage levels to groups of six healthy mice, weighing between 13 and 17 g, to determine acute toxicity. The animals were observed for 7 days following administration and mortality was recorded. It was found that when death resulted, it occurred within 24 hours after dosing. Those animals which survived this period recovered fully with no apparent cumulative or residual effects. The LD_{50}'s determined by Fujimura and Okamoto are as follows (32):

Sample	LD_{50} (mg/kg)	
	i.p.	p.o.
DPG	1400	8100
DAG	1250	9600

Finney et al. investigated the acute toxicity of glycyrrhetic acid using albino mice of both sexes weighing between 18 and 22 g. When the acid was given orally or subcutaneously, no deaths occurred following single doses as high as 610 mg/kg, which was the maximal dose that could be administered by these routes. For intraperitoneal administration an LD_{50} of 308 mg/kg was determined. High doses caused sedation, palor of the extremities, and respiratory depression. Death was usually delayed, the mice generally dying on the second day after administration. Pathological examination revealed some evidence of peritonitis (26).

Using mice, Klosa determined the following LD_{50} values for potassium glycyrrhizinate: i.v. - 412 mg/kg; i.m. - 695 mg/kg; s.c. - 697 mg/kg; p.o. 1220 mg/kg (67).

Takagi and Harada examined the acute toxicity of Fm 100, an extract of licorice, using male mice weighing approximately 20 g. Following intravenous and intraperitoneal injections, LD_{50}'s of 251 and 641 mg/kg, respectively, were determined using groups of 10 and 60 mice. The oral administration of Fm 100 revealed no toxicity within the range which was technically possible (129).

One hour after pigeons received subcutaneous injections of glycyrrhizin amounting to 0.45 - 0.50 g/kg, Tocco observed abundant excretion of feces, first solid, then liquid, of yellow color, considerable depression and regurgitation of yellow-colored substances which, when treated with concentrated nitric acid, reacted like bile pigments. The depression and other disorders disappeared in about 24 hours (130).

Tocco found that the subcutaneous injection of glycyrrhizin at a level of 1 g/kg results in the death of guinea pigs in less than 24 hours. Approximately 30 minutes after injection the animals become torpid and inclined to sleep. A strong general depression follows, the cardiac pulsation number diminishes, urinary secretion becomes scarce and the excreted feces indicated diarrhea (130).

Glycyrrhizin was administered to dogs, by Tocco, in doses of up to 2 g/kg. Intravenous doses of 0.45-0.50 g/kg were found to be fatal. When administered subcutaneously the substance produced slight depressive symptoms (staggering walk, diminished respiration frequency, sleepiness) which vanished in 1 to 3 hours. Oral administration produced no other symptoms than some alvine discharge (130).

II. Short Term Studies

Rats

For a period of 50 days, Girerd et al. administered, intragastrically, 10 g of licorice/kg/day to 14 rats (Group B), gave 1 g of ammoniated glycyrrhizin/kg/day to 13 rats (Group C), and maintained 10 rats as controls (Group A). Male Sprague-Dawley rats averaging 223 g were employed. The animals were weighed every 3 days and their blood pressure was measured at the start of the experiment and on days 15, 25, 35, and 45. After 50 days all survivors were sacrificed and autopsied. The control rats exhibited a moderate rise in blood pressure to a maximum of 125 mm Hg, while the treated animals showed a progressive and pronounced rise, reaching a peak of 186 and 194 for Groups B and C, respectively. The daily intake of saline for Group C was similar to the control group, while Group B showed a significantly higher intake. Growth of the animals was similar in Groups A and C for the first 36 days, after which the rats in Group C failed to keep up with the controls. The growth of Group B paralleled the controls only for the first week and then showed a highly significant depression. The survival rate of the animals, was 100, 77, and 36% for Groups A, B, and C, respectively. Measurement of the organs revealed an increase in weight of the kidneys, adrenals, and heart and a decrease in the weight of the hypophyses and testes in the treated animals compared to the controls. Severe renal and cardiovascular lesions, consisting essentially of arteriolar necrosis and hyalinization, were found in the licorice treated rats. Milder lesions were found in the glycyrrhizin group while no lesions were found among the control animals. It was concluded that the hypertensive properties of licorice could be attributed to the glycyrrhizin content, while polydipsia and some other effects may be due to other constituents (39).

Macabies investigated the effects of glycyrrhizin on 30 male Wistar rats maintained on various diets. Lot 1 (6 rats) received a standard diet, Lot 2 (12 rats) received a low-protein diet (protein 12.3%, lipids 15.7%, carbohydrate 72.%), and Lot 3 (12 rats) received the same low-protein diet supplemented with a beverage with a strong alcoholic content (96° ethanol 15 ml, water g.s.p. 100 ml). All of the animals received, per os, 160 mg of glycyrrhizin per day for a period of 70 days, followed by a period of 50 days during which no glycyrrhizin was administered. From this

point on, all of the animals were given the standard diet. Glycyrrhizin was again administered daily, at the same level, for another 35 days, after which the animals were observed for 20 days. Determinations of body weight and blood pressure were made twice monthly. No effect on weight was noted but a significant increase (25%) in blood pressure was observed in all of the animals during glycyrrhizin administration. When glycyrrhizin treatment was terminated, the blood pressure of the animals returned to the initial level. The second period of glycyrrhizin administration produced the same results (86).

In order to determine the hypertensive action of various compounds prepared from licorice, Macabies et al. administered, to groups of 5-10 male Wistar rats for a period of 10-25 days, the following: intraperitoneal ammoniated glycyrrhizin: 150 mg/kg and 300 mg/kg; subcutaneous ammoniated glycyrrhizin: 300 mg/kg; intraperitoneal tripotassium glycyrrhizin: 150 mg/kg and 300 mg/kg; "deglycyrrhizinated" licorice extract per os: 800 mg/kg; alpha and beta glycyrrhetinic acid: 300 mg/kg. All of the salts of glycyrrhizic acid exhibited a hypertensive action; discontinuation of the treatment resulted in a return to normal of arterial blood pressure. The isomers of glycyrrhetinic acid also exerted a strong hypertensive action while the "deglycyrrhizinated" extracts, containing 3-4% glycyrrhizin, showed only a very weak hypertensive action (85).

For a period of 90 days, Fujimura and Okamoto fed 10 groups of 5 healthy Wistar rats, 5 groups of males and 5 of females, di-potassium glycyrrhizinate (DPG) and di-ammonium glycyrrhizinate (DAG) at dietary levels of 0, 0.1, and 0.5%. The body weight of the rats and the amount of food consumed were measured daily. After 90 days, the animals were sacrificed and the liver, kidneys, spleen, and adrenals were weighed and examined microscopically. At the higher dosage levels of both DPG and DAG, the male rats exhibited smaller increases in body weight gain than did the controls; the females were unaffected. The weights of the internal organs were found to be unaltered and histological and pathological examination failed to reveal any untoward effects attributable to the glycyrrhizinates (32).

Klosa administered potassium glycyrrhizinate to rats (unspecified number) daily at a level of 60 mg/kg for a period of 8 months. Determination of the weight of the animals every third week disclosed no abnormal weight increases. The behavior of the treated animals did not differ from that of the controls (67).

Man

Molhuysen et al. administered daily to 10 human subjects, 20-45 g of succus liquiritiae, a dried watery extract of the roots of Glycyrrhiza glabra containing 15% glycyrrhizin. Retention of water, sodium, and chloride resulted from ingestion of the extract. Hemoglobin and total serum protein decreased; venous pressure, blood pressure, and pulse pressure rose considerably, and the output of potassium was much increased (94).

Following the oral administration of ammonium glycyrrhizinate (as much as 6 g/day for 3 days or 4 g/day for 5 days) to 7 normal people, 2 adrenogenital and 1 Cushing's syndrome patients, Louis and Conn concluded that ammonium glycyrrhizinate administration greatly enhances retention of sodium, chloride, and water and causes a mild increase in urinary potassium. They found that the concentration of sodium and chloride in thermal sweat are sharply lowered by glycyrrhizin. A significant decrease of 17-ketosteroids was noted, indicating inhibition of the pituitary-adrenal system. The ability of glycyrrhizin to inhibit pituitary release of MSH (melanocyte-stimulating hormone) was also noted. However, there were no discernible changes in organic metabolism (protein, carbohydrate, uric acid, creatinine, or circulating eosinophil) (84).

Block juice, a dried aqueous extract of licorice, was given by Card et al. to 2 healthy adult male subjects for 2 periods of 4 days each at levels of 20 and 36 g daily. Fluid intake, urinary output and chloride content were measured for 24-hour periods. Sodium and potassium were determined in an aliquot of the bulked urine for each period. Stools were collected and their electrolyte content measured separately. Results of the tests indicate that ingestion of the extract caused a gain in body weight, accompanied by a more positive fluid balance and a fall in urinary sodium and chloride excretion. A slight rise of both systolic and diastolic pressures was noted during the licorice periods (12).

After having eaten 700 g of licorice candy within a period of 9 days, a 53-year-old man suffered shortness of breath, ankle edema, increasing abdominal girth, weight gain, headache, and weakness for one week. He was admitted to a hospital where physical examination revealed a well-developed man in moderate respiratory distress. His blood pressure was 160/80 mm Hg and an ECG showed flattened T waves and prominent U waves, suggestive of hypokalemia. With a 2 g sodium diet and bed rest, the patient lost 12 lbs over the next four days and his blood pressure and ECG returned to normal. He was then discharged, to receive spironolactone, 25 mg 3 times a day for one week. A follow-up examination 3 weeks later gave all normal results (14).

As a self-prescribed treatment for an ulcer, a 48-year-old man consumed a 35 g licorice bar every other day for a period of approximately 6 months. During this time the subject experienced increased cardiac sensations, agitation in the heart area, throbbing of the pulse at the temples, and a feeling of oppression. After 6 months professional help was sought and a systolic blood pressure of 230 was determined. Discontinuance of licorice consumption, along with a salt-free diet, bed rest, and mild laxative and sedation therapy resulted in return of the blood pressure to normal within 14 days (51).

Rouques reported examining 5 cases of intoxication resulting from the consumption of antesite, a drink flavored with a licorice extract. These cases were characterized by hypertension and polydipsia. Potassium was found to be diminished in only one case. In all cases, recovery followed the elimination of glycyrrhizin (117).

Chronic edema of the legs and ankles of a 19-year-old woman was reported by Crampton. He found that the edema was caused by consumption of large amounts of licorice candy and that continued abstinence from licorice resulted in rectification of the edema (17).

Two cases of glycyrrhizin intoxication were reported by Potton et al. They found that the syndrome includes constant arterial hypertension, sometimes severe neuromuscular symptoms with occasional tetanic manifestations and a biological syndrome characterized by metabolic alkalosis with severe hypokalemia and hypernatremia. Intoxication developed only when 1 g/day or more was ingested. Total regression of the syndrome occurred within 20 days of cessation of ingestion (113).

Froment et al. reported the case of a 35-year-old man hospitalized for arterial hypertension and arterial obliteration of the lower limbs. Knowledge of the patient's consumption of approximately 1 g of glycyrrhizin per day (in the form of Antesite) led to testing for a possible association between the glycyrrhizin intake and the hypertension. No such relationship was found. However, laboratory tests revealed increased aldosteronuria during consumption of the glycyrrhizin with decreased plasma renin activity (30).

III. Long term

None

IV. Special studies

Teratologic

Ammonium glycyrrhizinate was administered daily by oral intubation to 109 pregnant adult female albino CD-1 outbred mice at levels of 0, 27, 90, 300, and 1000 mg/kg beginning on the 6th day and continuing through the 15th day of gestation. On day 17 all of the animals were subjected to Caesarean section and the numbers of implantation sites, resorption sites, and live and dead fetuses were recorded. Detailed examination of the urogenital tract of each dam was performed and the fetuses were examined for any abnormalities. It was concluded that ammonium glycyrrhizinate, administered under the specified conditions exhibited no teratological effect nor unfavorably influenced maternal or fetal survival (28).

Ammonium glycyrrhizinate was administered daily by oral intubation to 106 pregnant adult female albino rats (Wistar derived stock) at levels of 0, 27, 90, 300, and 1000 mg/kg beginning on the 6th day and continuing through the 15th day of gestation. On Day 20 all of the animals were subjected to Caesarean section and the numbers of implantation sites, resorption sites, and live and dead fetuses were recorded. Detailed examination of the urogenital tract of each dam was performed and the fetuses were examined for any abnormalities. It was concluded that ammonium glycyrrhizinate administered under the specified conditions exhibited no teratological effect nor unfavorably influenced maternal or fetal survival (28).

Ammonium glycyrrhizinate was administered daily by oral intubation to 111 pregnant adult female golden hamsters at levels of 0, 27, 90, 300, and 1000 mg/kg beginning on the 6th day and continuing through the 10th day of gestation. On Day 15 all of the animals were subjected to Caesarean section and the numbers of implantation sites, resorption sites, and live and dead fetuses were recorded. Detailed examination of the urogenital tract of each dam was performed and the fetuses were examined

for any abnormalities. It was concluded that ammonium glycyrrhizinate administered under the specified conditions exhibited no teratological effect nor unfavorably influenced maternal or fetal survival (28).

Ammonium glycyrrhizinate was administered daily by oral intubation to 53 pregnant adult female Dutch-belted rabbits at levels of 0, 27, 90, 300, and 1000 mg/kg beginning on the 6th day and continuing through the 18th day of gestation. On Day 29 all of the animals were subjected to Caesarean section and the numbers of implantation sites, resorption sites, and live and dead fetuses were recorded. Detailed examination of the urogenital tract of each dam was performed and the fetuses were examined for any abnormalities. It was concluded that ammonium glycyrrhizinate administered under the specified conditions exhibited no teratological effect nor unfavorably influenced maternal or fetal survival (28).

Biochemical Aspects

I. Break down

No information available

II. Absorption - Distribution

No information available

III. Metabolism and Exertion

By measuring the radioactivity of the urine, feces, blood, and bile resulting from oral administration of tritium-labelled monoammonium glycyrrhizinate (MAG), Carlat et al. determined that MAG is only slightly absorbed by the human gastrointestinal tract, 98% of the radioactivity being in the feces within 48 hours after administration. By measuring the radioactivity of the bile and blood, the possibility of MAG being absorbed into the blood and excreted via the bile was dismissed. Isolation of glycyrrhetic acid (GA) from the feces indicates that MAG is hydrolyzed in the body to the free GA, although the extent of hydrolysis was not determined. Other radioactive materials were also isolated from the feces but were not identified. Oral administration of labelled GA produced essentially the same results (13).

To determine the fate of glycyrrhetic acid, Parke et al. administered tritium-labelled beta-glycyrrhetic acid to both male and female albino rats. The results obtained are shown in the following table:

Most of the radioactivity was excreted in the feces, only traces being found in the urine. Labelled beta-glycyrrhetic acid was also administered, both orally and i.p. in doses of 25 mg/kg, to rats with biliary cannulae and it was found that most of the radioactivity was excreted in the bile. Three metabolites of beta-glycyrrhetic acid were determined in the bile, but were not identified (106).

Analysis of the urine of 2 patients given, orally, 2.28 and 2.5 g, respectively, of glycyrrhetic acid daily failed to show any of the unaltered compound. Further investigations indicated that a metabolite

of the acid was present in the urine, but it has not yet been identified.

IV. Effects on Enzymes and Other Biochemical Parameters

In vitro experiments by Whitehouse et al. have shown that glycyrrhetic acid is a potent uncoupler of oxidative phosphorylation in rat liver mitochondria (139).

In vitro tests by Noda indicate that glycyrrhizin inhibits the enzymatic activity of proteinase and of a homogenate prepared from dog spleen (102).

After receiving 0.4% ammoniated glycyrrhizin as drinking water for 1 week, 5 rats were fasted for 48 hours. Samples of blood taken from the rats at 0, 24, and 48 hours indicated that glycyrrhizin decreased the ability of the rats to mobilize glucose, thereby suggesting that glycyrrhizin decreases the output of ACTH (69).

Kraus exposed 10 untreated and 10 treated male mice (8-10 g) to a temperature of 5° for 8 hours. The treated mice had received 0.4% ammoniated glycyrrhizin in place of drinking water for 4 days prior to stress. The glycyrrhizin-treated mice died more rapidly than did the controls, 2.6 hours compared to 6.5 hours, suggesting that glycyrrhizin depresses the output of ACTH (69).

Gujral et al. reported that glycyrrhizan does not prolong the survival time of adrenal ectomised rats. Nor does it effect the absolute or relative weights of the spleen or adrenals or cause any depletion of the ascorbic acid and cholesterol levels of the adrenal glands. It produces no effect on the total white blood cells or circulating lymphocyte and eosinophil count (48).

Eudokimova and Kamilov found that potassium glycyrrhizinate shows a positive effect on the course of experimental atherosclerosis in rabbits. It decreases the amount of cholesterol in the blood and reduces the cholesterol-lecithinic coefficient (24).

Gujral et al. found that glycyrrhizin exhibits antiarthritic and anti-inflammatory effects in Brownlee's formaldehyde-induced arthritis in adrenalectomised rats (47).

By oral administration to bilaterally adrenalectomized rats, Hassan et al. determined that ammoniated glycyrrhizin, monoammonium glycyrrhizinate, and glycyrrhetic acid significantly decreased sodium output and caused retention of urine. Ammoniated glycyrrhizin and monoammonium glycyrrhizinate had little or no effect on potassium output, but glycyrrhetic acid increased K retention (52).

Following the administration of as much as 500 mg of glycyrrhetic acid to male albino rats daily for 8 days, Linko and Vasama noted a decrease in the excretion of sodium and on increase in the excretion of potassium. At the same time the body weight of the rats was found to

increase. It was found that glycyrrhetic acid has a desoxycorticosterone-like effect on the zona glomerulosa, and also to some extent of the transitional zone of the adrenals. Inhibition of the pituitary-adrenal system was attributed to the glycyrrhetic acid (81).

In cats and rats, Finney et al. found that glycyrrhetic acid exhibits a marked antidiuretic action and causes retention of sodium and an increase in urinary potassium excretion (26).

Using male albino rats, Galal found that, when given by any route, glycyrrhetic acid exhibits a strong antidiuretic effect and, when given orally, delays water absorption from the alimentary tract (33).

Card et al. gave 3 human subjects glycyrrhetic acid in daily doses of 0.2, 0.25, and 0.5 g, respectively, for 4 days. They found that glycyrrhetic acid had a retaining effect on water, sodium, and chloride (12).

Oral administration of 2 g of glycyrrhizic acid daily was found by Revers to produce edema in human subjects (115).

Over a period of years Revers investigated the effectiveness of extract of licorice in the treatment of gastric and duodenal ulcers. Three times per day patients were treated with one teaspoon of a preparation consisting of 100 g of powdered extract of licorice and 50 g of water. Control x-rays were taken every 2 or 3 weeks and the following results were obtained for 45 cases of gastric ulcers.

First control	-	ulcer disappeared	17 cases
First control	-	ulcer diminished	22 cases
First control	-	ulcer unchanged	6 cases
Second control	-	ulcer disappeared	11 cases
Second control	-	ulcer still present	17 cases
Third control	-	ulcer disappeared	1 case

Treatment of duodenal ulcers did not show as great a percentage of favorable results (115).

The influence of extract of licorice on intestines of animals was studied using intestines suspended in Tyrode solution and on intestines left in situ. The effect of the extract on artificial gastric ulcers in rats was also studied. The following results were obtained. Extract of licorice reduces the intestinal tonus. It reduces the mobility of the intestines. It removes the strong muscular spasm of the intestine induced by acetyl chloride, histamine, or barium chloride. It can prevent experimental gastric ulcers in rats both in administering it by mouth and parenterally. It has no local anesthetical effect and no curarine action (115).

Nishiyama reported that the internal administration of licorice powder to 44 cases of peptic ulcer in human patients produced favorable results. The administration of licorice extract to shay rats was found to check the growth of ulcers while crude glycyrrhizin was without effect (101).

V. Drug Interaction

Sasano et al. reported that simultaneous i.v. administration of glycyrrhizin with dexamethasone to rats inhibits the dexamethasone-induced atrophy of the adrenals, indicating adrenocortical stimulation by the glycyrrhizin. It was suggested that the stimulant effect of glycyrrhizin on the adrenocortical cell is induced through the cortical capillary wall including the adrenal reticuloendothelial cell (121).

A series of experiments by Asanuma disclosed that glycyrrhizin suppresses the action of cortisone (inhibition of ACTH production) in adrenalectomized rats. The inhibition of complementary adrenal hypertrophy following unilateral adrenalectomy was also antagonized by glycyrrhizin to a significant degree. However, under histamine stress, glycyrrhizin intensified the action of cortisone. Glycyrrhizin was also found to suppress the inhibitory action of dexamethasone on the pituitary (4).

Gujral et al. reported that glycyrrhizin potentiates the antiarthritic action of hydroxycortisone in rats (47).

Elmadjian et al. found that monoammonium glycyrrhizinate and hydrocortisone have synergistic effects in the adrenalectomized patient (23).

Velluz reported that the soluble salts of glycyrrhizic acid are cryptotoxic for tetanus toxin (136).

VI. Consumer Exposure Information

Glycyrrhiza, ammoniated glycyrrhizin and licorice are used as a natural flavoring and a natural flavor extractive.

GLYCYRRHIZIN AND DERIVATIVES

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I. INTRODUCTION

It is generally known that glycyrrhizin, the main effective component of licorice root, possesses various biological activities. Its currently known biological actions include desoxycorticosterone-like or glucocorticoid-like action, the redintegration of gastric ulcers [1], the depression of plasma high cholesterol [2], and anti-allergic and detoxication actions. In relation to its mechanism of action, Kumagai et al. demonstrated that the inactivation of steroid in the liver could be suppressed by glycyrrhizin [3], and maintained that this suppressive activity was the basic form of its corticoid-like action. They also revealed that this compound antagonized the anti-granulation action of glucocorticoid [4].

However, there still remain many points of its mechanism that need explanation. In the present investigation, the effect of this compound on the adrenal system was studied to clarify the mechanism of its action. The study was also designed to elucidate the synthesis and secretion of ACTH and to pursue prevention of adrenal insufficiency due to medical treatment which is currently drawing clinical attention.

II. EXPERIMENTAL MATERIALS

1. EXPERIMENTAL ANIMAL

Male Wistar rats, weighing 200 - 250 g unless specified, were used. They were raised on Oriental solid feed at constant temperature for at

least one week before the experiment. The adrenals were removed from the dorsum under anesthesia. The animals were given 1% physiological saline solution in lieu of drinking water after bilateral adrenalectomy.

2. DRUGS AND REAGENTS

The following drugs and reagents were used: cortisone acetate (Schering Co.), prednisolone acetate (Scherine Co.), glycyrrhizin (Minophagen Institute), oxycellulose (11-12% COOH) (Tennessee Eastman Co), L-phenylalanine-U-C^{14} (The Radiochemical Center) (specific activity, 9.9 mc/mM), DL-phenylalanine-2-C^{14} (Daiichi Kayaku; specific activity, 2.4 mc/mM), acetone-dried pig anterior pituitary powder (Armour Co.). The dosages in this paper indicate the amounts per 100 g of body weight.

III. EXPERIMENTAL PROCEDURES

1. INCORPORATION OF C^{14}-PHENYLALANINE INTO C^{14}-ACTH AND TOTAL PROTEIN BY RAT ANTERIOR PITUITARY.

The animals, both normal and adrenalectomized, were treated with prednisolone, cortisone, and glycyrrhizin individually or in combination for 7 days from the day of adrenalectomy. The rats were decapitated 20 hours after the final injection. The anterior pituitaries were removed immediately without flaw, and their ACTH and protein producing capacity was examined by the procedure used by Wool et al. [5].

In the examination of ACTH synthesis, the pituitaries, 3 per container, were incubated with 2 cc of Krebs' Ringer bicarbonate buffer containing 2 mu of L-phenylalanine-U-C^{14} in the presence of a 95% O_2-5% CO_2 gas at 37°C for 3 hours.

After incubation, 1 g of acetone-dried, powdered pig anterior pituitary was added, with carrier ACTH, and the mixture was extracted from hot glacial acetic acid, purified by adsorption with oxycellulose.

The radioactivity of the ACTH extracted with L-phenylalanine-U-C^{14} was measured with Nuclear Chicago gas-flow counter, and the results were expressed in c.p.m./100 mg of anterior pituitary (wet wt).

In order to determine the protein production capacity, 5 rat anterior pituitaries were incubated with 2 cc of Krebs' Ringer bicarbonate buffer containing 1 uc of DL-phenylalanine-2-C^{14} at 37°C for 2 hours in the presence of a gas containing 95% O_2 and 5% CO_2. After incubation, the radioactivity in the residue of the pituitary after trichloroacetic acid treatment was measured with a gas-flow counter, and after self-absorption compensation, the results were expressed in c.p.m./mg of protein.

2. IDENTIFICATION OF THE EXTRACTED RADIOACTIVE SUBSTANCES WITH ACTH

The rats were decapitated on the 7th day after bilateral adrenalectomy, and the 60 anterior pituitaries removed from the animals were incubated with L-phenylalanine-U-C^{14}, and, after ACTH was extracted and purified by the above-described procedure, they were subjected to horizontal starch block electrophoresis under the following conditions: starch block. 3 x 40 cm; veronal-sodium veronal buffer, pH 8.6; ionic intensity, 0.05; 3 mA, 600 V; 13 hours.

After electrophoresis, each block was perfused with 0.1 N HCL, and the radioactivity, protein level and ACTH activity of the perfusate were measured. The quantitative determination of proteins followed the procedure of Lawry reaction [6], and the ACTH bioassay, a modified Hedner-Rerup's procedure [7] using ascorbic acid depletion method with steroid-blocked rats. In the latter, male rats weighing 100 - 130 g were treated by hypodermic injection of 5 mg prednisolone, and the test material was injected intravenously to the rats 4 hours later. The

rats were decapitated one hour later, and the left adrenal was removed to measure the ascorbic acid level. The quantitative determination of ascorbic acid followed Roe-and Kuether's procedure [8]. The authors also observed a linear relationship between the amount of decrease in ascorbic acid and the dose of ACTH within a range of 1.6 to 12.5 mU of ACTH (logarithmic scale) [9].

3. COMPLEMENTARY ADRENAL HYPERTROPHY FOLLOWING UNILATERAL ADRENALECTOMY

The right adrenals of male Wistar rats weighing 120 - 150 g were removed, and after 10 days of treatment with cortisone or glycyrrhizin or combined treatment with both from the day of operation, the rats were decapitated, and the left adrenals were removed. The adipose tissues around the excised adrenals were cleaned off, and the organs were weighed with a torsion balance to an accuracy of 0.1 mg. The following formula was used to calculate the rate of increase in the weight of one adrenal for that of the other.

$$\frac{\text{Weight of left adrenal - weight of right adrenal}}{\text{Weight of right adrenal}} \times 100$$

4. DEPLETION OF ADRENAL ASCORBIC ACID DUE TO HISTAMINE STRESS

Glycyrrhizin was intramuscularly injected to male rats weighing 120 - 150 g at daily dosage of 10 mg for three days, and cortisone, 2.5 mg, was included in the final injection. Four hours after the final injection, histamine chloride, 0.5 mg, was injected to the rats intraperitoneally, and the left adrenals were removed one hour later, in order to measure the amount of ascorbic acid.

5. CLINICAL EXPERIMENTS

Twenty patients with mild tuberculosis, who appeared almost normal

from the blood sedimentation rate and the results of kidney and liver function tests, were treated with dexamethasone at daily oral dosage of 1 - 2 mg for 3 days. The total urinary 17-OHCS was measured for 6 days consecutively from the day of treatment. At the same time, glycyrrhizin was administered orally along with the above drug at daily dosage of 150 ~ 300 mg for 6 days, and the total urinary 17-OHCS was measured. The quantitative determination of 17-OHCS, performed after extraction with butanol, followed Watanabe's procedure [10], which is a modified version of Porter-Silver reaction.

IV. EXPERIMENTAL RESULTS

1. EFFECTS OF ADRENALECTOMY AND PREDNISOLONE TREATMENT ON THE INCORPORATION OF C^{14}-PHENYLALANINE INTO ACTH AND GENERAL PROTEINS.

As reported by Wool et al. [5], adrenalectomy performed one week in advance increased the incorporation of L-phenylalanine-U-C^{14} into ACTH in vitro by 1.6 - 2.3 times (Table 1), but not the incorporation of DL-phenylalanine-2-C^{14} into general proteins (Table 2).

Table 1. Effect of adrenalectomy and prednisolone administration on incorporation of radioactivity from L-phenylalanine-U-^{14}C into corticotropin by isolated rat anterior pituitary

Exp. No.	Exp. Group	Radioactivity Incorporated Into Corticotropin Count/Min./100Mg Anterior Pituitary	Ratio	P†
1.	Normal (Saline only)	403±46* (5)	100	
	Adrenalectomized (Saline)	643±76 (5)	160	<0.05
	Prednisolone Treated	233±33 (5)	58	<0.05
2.	Normal	412±24 (9)	100	
	Adrenalectomized	939±111 (4)	228	<0.001

*Standard error of the mean. Number of observations in parenthesis.
† Compared with normal value.

After normal rats were treated with prednisolone at daily dosage of 5 mg per animal for 7 days, the ACTH producing capacity of rat pituitary dropped by 60% from the control level (Table 1).

Table 2. Effect of adrenalectomy on incorporation of radioactivity from D, L-phenylalanine-2-^{14}C into protein of isolated rat anterior pituitary gland

Exp. Group	Radioactivity Incorporated Into Protein Connt/Min./Mg Protein
Normal	1790 ± 224* (5)
Adrenalectomized	1834 ± 184 (5)

* Standard error of the mean. Numer of observations in parenthesis. The difference is not significant.

2. IDENTIFICATION OF EXTRACTED RADIOACTIVE MATERIALS WITH ACTH

Oxycellulose does not possess specific capacity to adsorb ACTH, but also adsorbs other basic peptides such as melanocyte stimulating hormone, etc. Thus, it was necessary to confirm the fact that the radioactive substance actually belonged to the ACTH fraction.

Horizontal starch block electrophoresis was employed for the identification of the extracted radioactive material. As shown in Figure 1, the radioactivity reached peaks at -1 cm and -6 cm from the original spot, which are in agreement with the peaks in the protein determination by Lawry reaction. Moreover, marked ACTH activities were observed in coincidence with these two peaks.

3. EFFECTS OF CORTISONE ON C^{14}-ACTH PRODUCTION STIMULATED BY ADRENALECTOMY

After bilateral adrenalectomy, cortisone was administered intramuscularly to the rats daily for 7 days at 0.1, 0.5, 1, and 5 mg/day, and the ACTH production of their pituitaries was studied.

As shown in Figure 2, the stimulation of C^{14}-ACTH secretion by the anterior pituitary of adrenalectomized rats could not be influenced by cortisone at up to 0.5 mg, but cortisone at 1 m inhibited the activity by a significant 60%. At 5 mg, the activity was inhibited to a more notable degree.

4. EFFECTS OF GLYCYRRHIZIN ON THE INHIBITORY ACTION OF CORTISONE ON C^{14}-ACTH PRODUCTION

Adrenalectomized rats were treated with cortisone at daily dosage of 1 mg, then with glycyrrhizin at 0.1, 1, and 10 mg per day in addition to cortisone.

Fig. 1. Horizontal starch block electrophoresis of the radioactive substance extracted by the oxycellulose procedure. The solid line indicates the protein content, expressed as the optical density at 750mμ determined by the Lowry reaction. The dotted line indicates the counts per minute of radioactivity determined with a Nuclear Chicago gas flow counter.

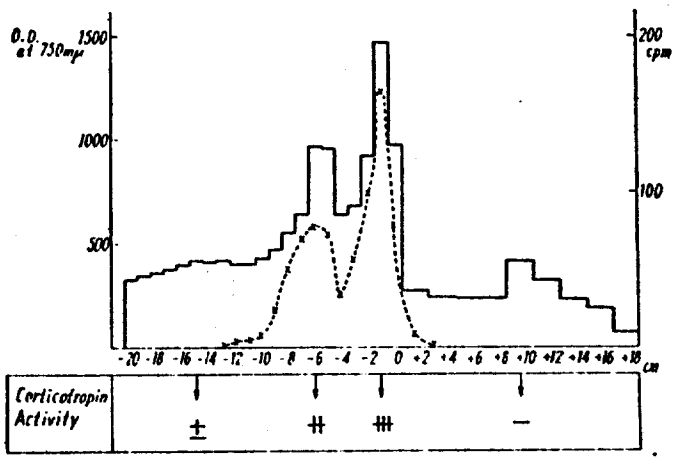
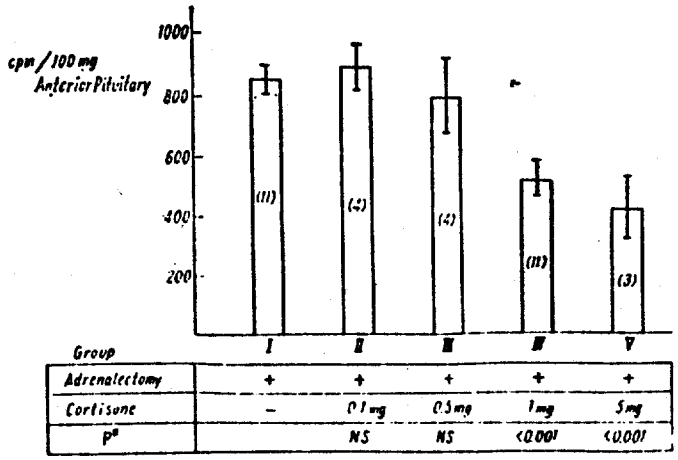


Fig. 2. Effect of cortisone on the increased incorporation of radioactivity from L-phenylalanine-U-^{14}C into ACTH by isolated anterior pituitary of rat induced by adrenalectomy. The vertical bars represent the standard error of the mean. The number of observations is in parenthesis.
* compared with group I



Subsequently, cortisone was administered to adrenalectomized rats at daily dosage of 2 mg, then with glycyrrhizin at 0.2, 2, and 20 mg/day in addition to cortisone, and the effects of glycyrrhizin on the inhibitory action of cortisone on C^{14}-ACTH production were studied. The results are shown in Figures 3 and 4. The production of C^{14}-ACTH, which had been stimulated by adrenalectomy, was suppressed by cortisone treatment to significant degrees, and in the cortisone-1 mg group, glycyrrhizin exhibited notable antagonism against cortisone at 0.1 - 10 mg.

At 2 mg/day of cortisone, its inhibitory action on C^{14}-ACTH production could be blocked by glycyrrhizin at significant rates only at 20 mg. Dosages of 0.2 - 2 mg/day of glycyrrhizin were insufficient to attain its antagonism against cortisone. Glycyrrhizin by itself showed no influence on the C^{14}-ACTH production in adrenalectomized rats (Figure 3) or intact rats (Figure 5).

5. EFFECTS OF GLYCYRRHIZIN ON THE INHIBITORY ACTION OF CORTISONE ON THE COMPLEMENTARY ADRENAL HYPERTROPHY FOLLOWING UNILATERAL ADRENALECTOMY

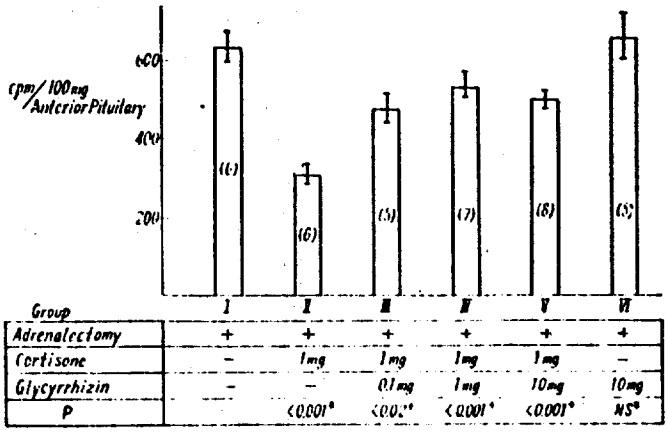
After unilateral adrenalectomy, cortisone was administered to rats at 1 mg/day, and subsequently glycyrrhizin was administered in addition at 0.1, 1, and 10 mg. The rate of increase in the weight of intact adrenal was 50% for the control group as shown in Figure 6. (The actual increase rate is considered to be 40%, for the left adrenal was 10% heavier than that of the right side) Cortisone at 1 mg/day controlled complementary hypertrophy completely. This inhibitory action was blocked to a significant degree by the combined use of glycyrrhizin at 10 mg. Glycyrrhizin alone showed no effect on complementary adrenal hypertrophy.

6. EFFECTS OF GLYCYRRHIZIN AND CORTISONE ON THE DEPLETION OF ADRENAL ASORCASCORBIC ACID DUE TO HISTAMINE STRESS

Adrenal ascorbic acid was chosen as the indicator of ACTH secretion due to stress, and the effect of glycyrrhizin on the activity was investigated.

A single cortisone treatment at 2.5 mg or 3-day glycyrrhizin treatment at daily dosage of 10 mg failed to block the depletion of adrenal ascorbic acid due to histamine stress. However, combined administration of cortisone and glycyrrhizin blocked the decrease to a significant degree (Figure 7)

Fig. 3. Effect of glycyrrhizin on the suppressive action of cortisone on the incorporation of radioactivity from L-phenylalanine-U-^{14}C into ACTH by isolated anterior pituitary of rat. The vertical bars represent the standard error of the mean. The number of observations is in parenthesis.
* compared with group I
† compared with group II



7. CLINICAL RESULTS

As shown in Figure 8, dexamethasone suppressed pituitary activity and consequently reduced urinary 17-OHCS, the rate of decrease being higher with larger dosage. When the treatment was withdrawn, the 17-OHCS level became normal. The combined administration of dexamethasone and glycyrrhizin suppressed the inhibitory effect of the former completely in 11 cases out of 20, retarded the inhibition in 3 cases, and showed no

effect in 6 cases. Figure 8 illustrates the clinical results of typical cases.

Fig. 4. Effect of glycyrrhizin on the suppressive action of cortisone on the incorporation of radioactivity from L-phenylalanine-U-^{14}C into ACTH by isolated rat anterior pituitary. The vertical bars represent the standard error of the mean. The number of observations is in parenthesis.
* compared with group I
† compared with group II

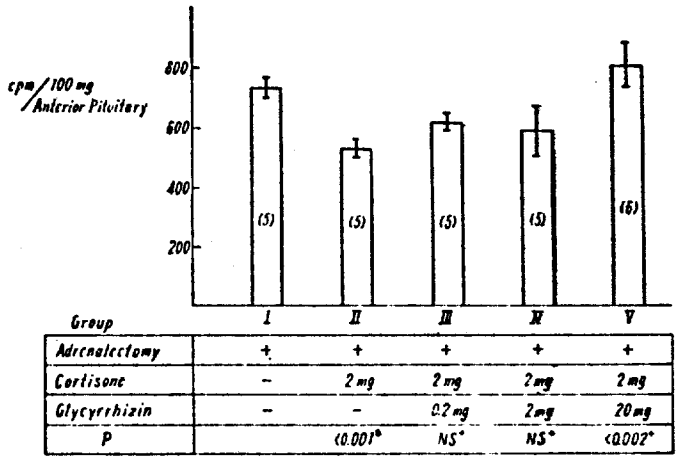


Fig. 5. Effect of glycyrrhizin on the incorporation of radioactivity from L-phenylalanine-U-^{14}C into ACTH by isolated anterior pituitary of rat. Vertical bars indicate the standard error of the mean. The differences between the control value and those of the various experimental groups are not significant.

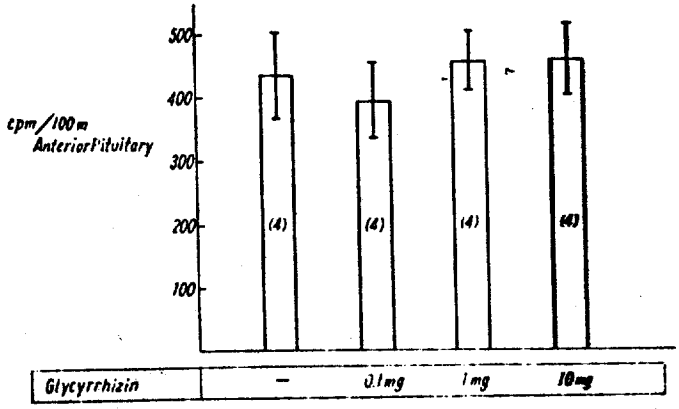
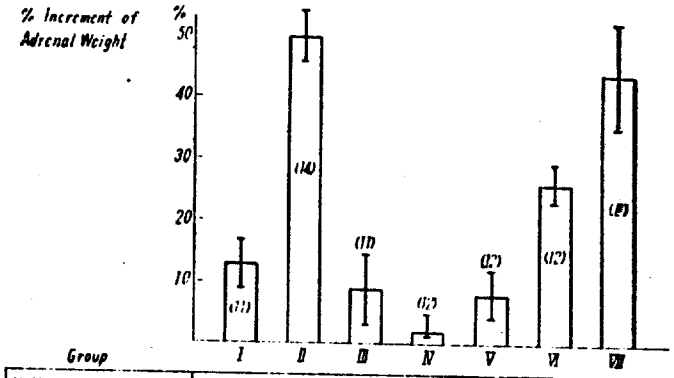
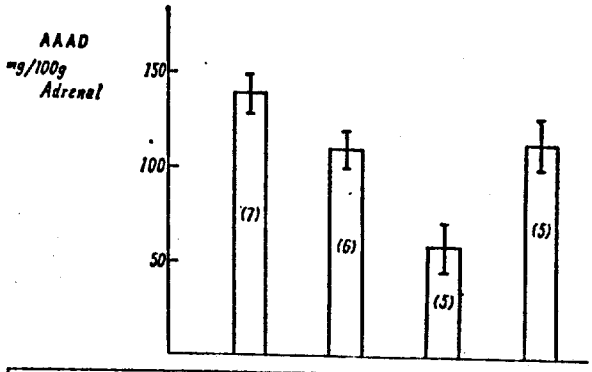


Fig. 6. Effect of glycyrrhizin on the suppressive action of cortisone on compensatory adrenal hypertrophy after unilateral adrenalectomy. Vertical bars indicate the standard error of the mean. The number of observations is shown in parenthesis.
* compared with group I
† compared with group II
§ compared with group III



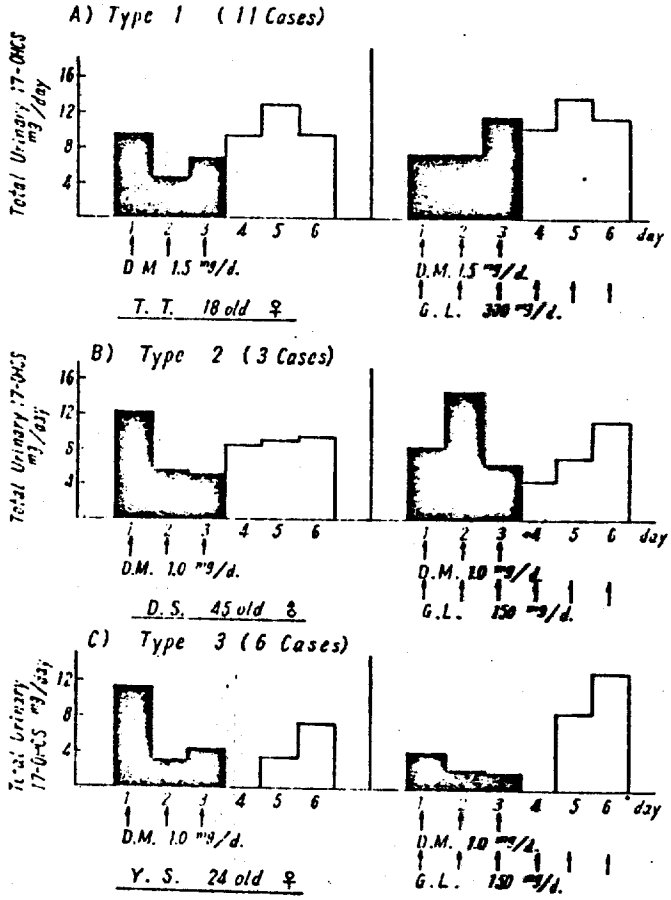
Unilateral Adrenalectomy	–	+	+	+	+	+	+
Cortisone	–	–	1mg	1mg	1mg	1mg	–
Glycyrrhizin	–	–	–	0.1mg	1mg	10mg	10mg
P		<0.001*	<0.001*	NS§	NS§	<0.05§	NS*

Fig. 7. Effect of cortisone and glycyrrhizin on adrenal ascorbic acid depletion of the rat after histamine stress. Vertical bars indicate the standard error of the mean. The number of observations is in parenthesis.
* compared with control group



Cortisone	–	2.5mg	2.5mg	–
Glycyrrhizin	–	–	10mg	10mg
P*		NS	<0.02	NS

Fig. 8 Effect of glycyrrhizin on the action of dexamethasone in decreasing the amount of total urinary 17-OHCS.
Type 1 shows the case in which glycyrrhizin effectively blocked the pituitary inhibition by dexamethasone.
Type 2 shows the case in which glycyrrhizin delayed the pituitary inhibition by dexamethasone.
Type 3 shows the case in which glycyrrhizin had no effect on the pituitary inhibition by dexamethasone.
D.M. : dexamethasone G.L. : glycyrrhizin



V. DISCUSSION

In order to clarify the biological activities of glycyrrhizin, the effects of this compound on the pituitary and adrenal system was investigated. The experiment demonstrated that glycyrrhizin antagonized the inhibitory action of cortisone on the pituitary and adrenal activities. The compound also exhibited strong antagonism against cortisone in its inhibition of pituitary C^{14}-ACTH production and complementary adrenal hypertrophy following unilateral adrenalectomy. Furthermore, glycyrrhizin

was found to be antagonistic against the pituitary inhibition of dexamethasone in the clinical experiment using urinary 17-OHCS as an indicator.

It is already known that glycyrrhizin inhibits the metabolism of steroid having Δ^4 3-ketone in the liver and enhances the serum level of biological, active steroid [3]. The elevation of the active serum glucorticoid level should consequently inhibit the pituitary function, i.e., it should suppress the synthesis and secretion of ACTH. In the present experiment, however, glycyrrhizin blocked the pituitary inhibition by glucocorticoid. The reason for this unexpected result is interpreted as follows.

Glycyrrhizin is assumed to antagonize the feedback action of glucocorticoid at the site subject to the action - probably the diencephalon, and its antagonistic action exceeds in degree the diencephalic pituitary inhibition by the active serum glucocorticoid which had been enhanced by the inhibited steroid metabolism in the liver by glycyrrhizin.

The following interpretation may also be permitted. The dosage of glycyrrhizin used in this experiment was relatively small. Lawrence et al. [11] administered glycyrrhizin orally at 4 - 6 g to human, and found that, with the decrease in urinary 17-KS as an indicator, the compound exhibited moderate pituitary inhibition. In many of the experimental reports which demonstrate the presence of DOC-like action in glycyrrhizin, the dosage is considerably large as in Lawrence's experiment. On the contrary, the dosage in this experiment ranged from 150 to 300 mg for human and 0.1 to 20 mg for rat. The effects of this compound on the steroid metabolism in the liver at such small dosage may be insignificant, and it may even be possible that, under this condition, the antagonism against the feedback action of glucocorticoid

may manifest itself before such effects.

Sayers and Sayers [12] proposed the negative feedback control theory, i.e., they maintained that the secretion of ACTH is controlled by the corticoid level in the circulating blood. In the present experiment, adrenalectomy stimulated specifically the pituitary ACTH production to a marked degree and the synthesis of ACTH was suppressed by prednisolone treatment. This experimental finding is one of the examples that directly substantiatethe negative feedback control theory.

Many investigators deny that the negative feedback mechanism participates in the secretion of ACTH due to stress [13-16]. This investigation also demonstrated that glycyrrhizin antagonized the feedback action of glucocorticoid at basal or resting stage, but it accelerated the action of cortisone under histamine stress. This may be indicative of the fact that the mechanism of ACTH production due to stress is different from the secretion mechanism at the basal stage.

Glycyrrhizin is a diglucopyranosiduronic acid of glycyrrhetinic acid, and its activity unit is glycyrrhetinic acid. This acid is a non-steroid triterpenoid, and has stereochemical resemblance to allo-pregnane. Its influences on the glucocorticoid action may be derived from its structural similarity to steroid. Such action of this compound may manifest itself by antagonistic inhibition.

VI. CONCLUSION

1. Pituitary ACTH production capacity in rat was stimulated specifically by adrenalectomy, and suppressed by the pretreatment with prednisolone. This directly supports the negative feedback control theory.

2. The stimulated pituitary ACTH production in adrenalectomized

rat was inhibited by cortisone treatment. The combined treatment with glycyrrhizin and cortisone suppressed the action of the latter. Similarly, the inhibition of complementary adrenal hypertrophy following unilateral adrenalectomy was also antagonized by glycyrrhizin to a significant degree.

3. In the clinical experiment using urinary 17-OHCS as the indicator, glycyrrhizin was found to suppress the inhibitory action of dexamethasone on the pituitary.

4. Under histamine stress, glycyrrhizin intensified the action of cortisone, and the ACTH secretion due to stress appeared to have different mechanism from normal activity.

Sincere gratitude is expressed to Prof. Yamamura for his guidance and encouragement. This experiment was assisted by Dr. Kumagai, Dr. Yano, and Dr. Takeuchi, to whom the authors are grateful. Acknowledgement is also due to the endocrinological study group of the Department of Endocrinology, including the instructors and Dr. Watanabe of the Central Laboratory.

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EFFECTS OF LIQUORICE AND ITS DERIVATIVES ON SALT AND WATER METABOLISM

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RECENT reports have suggested that some samples of liquorice and their extracts may possess previously unsuspected pharmacological activity.

Revers (1946, 1948, 1951) noticed that about 20% of patients taking liquorice developed œdema. Molhuysen et al. (1950) described retention of water, sodium, and chloride, with increased output of potassium, following the use of a dried aqueous extract of liquorice. This action they defined as deoxycortone-like, and we shall use the term in this sense throughout this paper. They treated one case of Addison's disease with this preparation, without apparent effect. Groen et al. (1951) claim to have controlled one case of Addison's disease with liquorice, and a further case with salts of glycyrrhizinic acid, a component of liquorice (1952). The effects described in these two patients resembled those of deoxycortone.

We decided to investigate these effects, and in addition we examined certain components of liquorice for deoxycortone-like activity.

CRUDE LIQUORICE IN NORMAL SUBJECTS

Methods

Two healthy male adult subjects (S. and W.) were investigated. The trial extended to six periods, each of four days. An initial control period was followed by two on liquorice, then a second control period. During the fifth period deoxycortone in oil was injected intramuscularly once daily, after which a final control period was included to compare the recovery from liquorice administration and from deoxycortone. Sodium and potassium were measured in the diet. This provided an intake of approximately 3200 calories daily, which was enough to prevent the subjects going into negative nitrogen balance, and thus losing water, potassium, and nitrogen from protein katabolism. Gelatin capsules of salt were taken as supplements to provide approximately 9 grammes of sodium daily in all. The analytical methods used were those described by King (1951).

Fluid intake, urinary output, and chloride content were measured for twenty-four-hour periods. Sodium and potassium were determined in an aliquot of the bulked urine for each period. Stools were collected and their electrolyte content measured separately. The "fluid balance" shown in figs. 1 and 2 takes account only of measured fluid intake and urinary output, without allowing for water of metabolism, food water, and water lost in the expired air and through the skin. The weight was taken daily before breakfast.

The liquorice used was part of a bulk sample of Turkish "block juice" set aside for clinical trial. "Block juice" is the commercial name for the dried aqueous extract of liquorice prepared by primitive methods. The roots of the plant are boiled in water and the solution is evaporated to dryness. As supplied to us, it consisted of small black granules with the characteristically sweet taste; it was made up in rice paper packages containing 4 g. each, and in this way was readily swallowed.

Results

Effects on blood-pressure, venous hæmatocrit values, hæmoglobin, weight, urinary chloride and specific gravity, and "fluid balance" are illustrated in figs. 1 and 2. Electrocardiograms showed no appreciable change in either subject while taking liquorice or after deoxycortone.

A third subject (C.) took 20 g. liquorice daily, but without attempting to control sodium and potassium balance, and showed a maximum gain in weight of 4 lb. in a four-day test period.

Discussion

Weight. The most striking effect of taking liquorice was a gain in weight. This was accompanied by a more positive "fluid balance" and a fall in urinary chloride excretion. In both subjects the "fluid balance" was more positive in period II than in any period throughout the trial. Deoxycortone produced a positive "fluid balance" in W. only, though not to the same extent as did liquorice. During period III neither subject gained any more weight, though in S. the dose of liquorice was almost doubled. Withdrawal of liquorice was followed by a diuresis and loss of weight, as shown in period IV.

The response to deoxycortone differed in the two subjects. In W. 5 mg. deoxycortone in oil injected intramuscularly once daily produced a gain in weight and a positive "fluid balance" appreciably less than that due to liquorice in period II. In S. deoxycortone injections were accompanied by a loss of weight. When the deoxycortone was stopped, the weight fell in both subjects, but this fall was accompanied by a diuresis in W. only. Judging by subsequent experience, it is probable that the period allowed for recovery from liquorice was too short for equilibrium to be regained.

Water distribution.—The "thiocyanate space" (Crandall and Anderson 1934) was measured in both subjects on day 5, before starting liquorice, and on day 12, the last day of taking liquorice. In both subjects two-thirds of the gain in weight was accounted for by an increase of the "thiocyanate space."

Blood-pressure.—In both subjects there was a slight rise of systolic and diastolic pressures during the liquorice periods, and in W. on deoxycortone.

Sodium and potassium balances.—These were not wholly satisfactory, but sodium balance approximately followed changes in urinary chloride, "fluid balance," and weight.

Sodium/potassium ratio.—The ratio of urinary sodium to potassium was calculated for each period. As a measure of deoxycortone-like activity this ratio is more sensitive than the change in the excretion of each ion taken by itself. A fall in the sodium-potassium ratio would then indicate deoxycortone-like activity in liquorice. In fact only a small fall was noted on liquorice, but even this was larger than was noted on deoxycortone.

Conclusions.—In general these findings confirm the claims of Molhuysen et al. (1950) except that we failed to demonstrate any certain effect on potassium excretion.

LIQUORICE FRACTIONS IN NORMAL SUBJECTS

Liquorice block juice was then fractionated by solvent and other treatment. A screening test was devised by which we hoped to identify fractions rich in the substance responsible for the effects described. The same three subjects were weighed daily. Urine was collected in twenty-four-hour specimens and chloride output measured. No attempt was made to control intake of salt or water, and it was hoped that, by taking the sum of four successive days together, the effect of variations in excretion due to changes in intake would be minimised. Alternate periods of four days were taken as control and test periods. The usual dose was the equivalent of 20 g. of block juice daily.

With the screening test described no definite evidence was found that any of the substances had had an effect. Where one of them seemed to have an effect in one of the subjects, it was not always observed in the others. This apparent inconsistency between subjects would not by itself invalidate the results; we have since satisfied ourselves that the subjects did show considerable variation in response, due apparently to differences in sensitivity.

Conclusion

We do not feel that the method used was adequate to identify an active fraction.

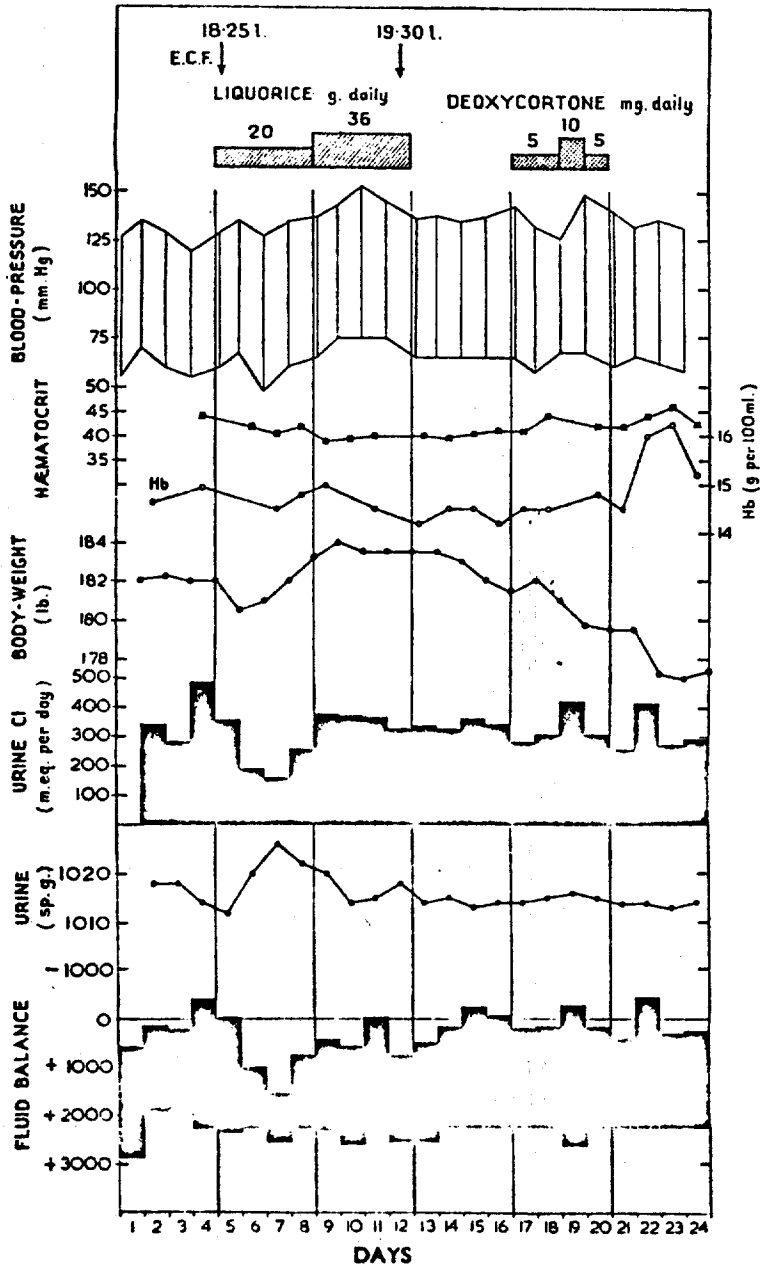

Fig. 1—Effect of crude liquorice and deoxycortone on daily blood-pressure, hæmatocrit, hæmoglobin, weight, urinary chloride, urinary specific gravity, and "fluid balance" in normal subject S. 4-day periods. E.C.F. = thiocyanate space.

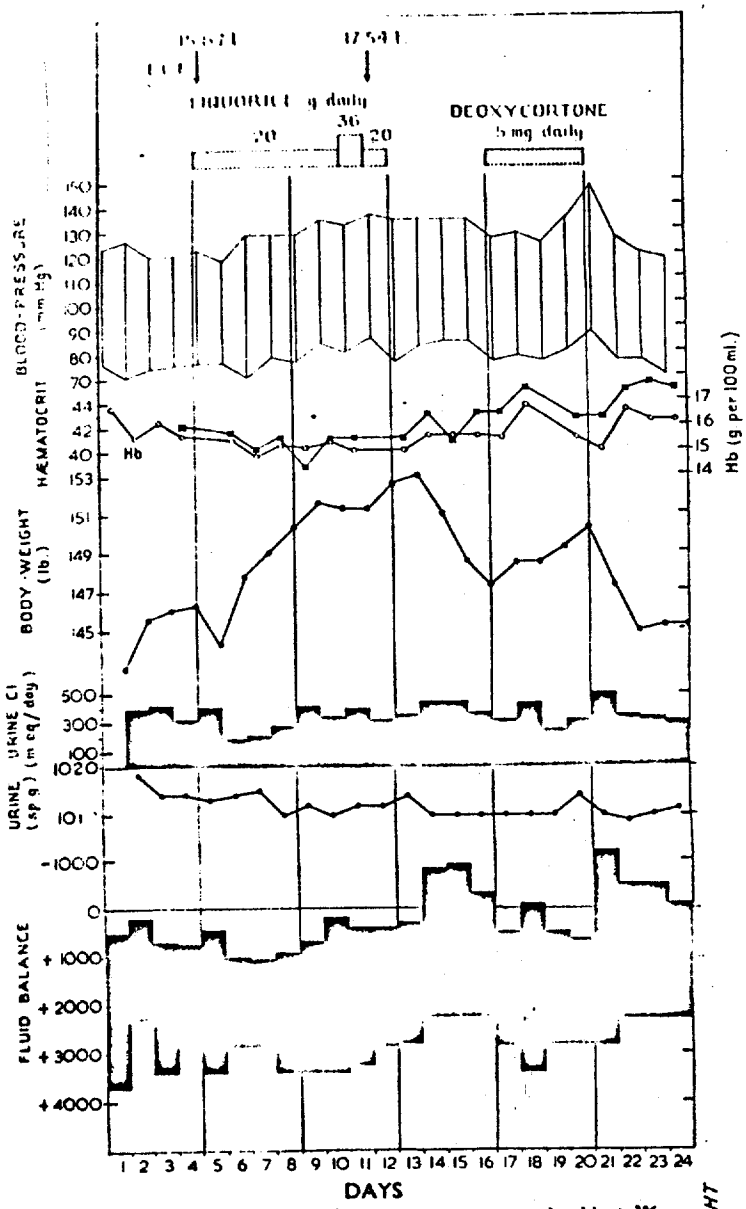

Fig. 2—Effect of crude liquorice and deoxycortone on normal subject W. 4-day periods.

GLYCYRRHETINIC ACID IN NORMAL SUBJECTS

Groen et al. (1952) suggested that the water-and-salt-active substance in crude liquorice might be glycyrrhetinic acid, which when conjugated with glucuronic acid forms glycyrrhizinic acid. This in turn forms 2–7% of different samples of crude liquorice, as its potassium and calcium salts (*B.P.C. 1949*). (The *U.S. Dispensatory 1947* quotes 5–10% glycyrrhizin in raw liquorice.) A sodium, potassium, and chloride balance was carried out to test this suggestion.

Method

The same three subjects took a diet of constant composition over five four-day periods. After two control periods, glycyrrhetinic acid was taken orally for the next period in doses respectively of 0·2, 0·25, and 0·5 g. daily. Two further control periods followed. Daily measurements were made of body-weight, urinary volume, and chloride output. Daily aliquots of the urine in each period were mixed, and sodium and potassium content determined. Daily blood-pressure was also recorded in two subjects, but no notable changes were observed.

Results

The findings in subject C. are shown in figs. 3 and 4. It will be seen that body-weight rose on glycyrrhetinic acid (0·5 g. daily), with a corresponding diminution of urinary volume and chloride output. The balance shows retention of sodium and chloride, but no alteration in potassium exchange. (The apparently positive sodium and chloride balances during the control periods can be explained as unmeasured losses of these ions in the fæces and sweat.) The findings in subjects W. and S. showed similar though less marked changes.

Conclusions

In the three subjects tested glycyrrhetinic acid had a retaining effect on water, sodium, and chloride, but its influence on potassium exchange was equivocal.

GLYCYRRHETINIC ACID IN ADDISON'S DISEASE

An opportunity arose to test the effect of glycyrrhetinic acid on a patient with Addison's disease.

The patient, a woman of 45, complained of periodic blackouts and dizziness for seven years. For three months she had noted weakness and nausea, but no vomiting. There was no past or family history of tuberculosis.

She was thin (95 lb.); her face, neck, and hands were deeply pigmented, including the palmar skin folds; and there was also pigmentation of the buccal mucosa. The blood-pressure was 108/70 mm. Hg on first examination, falling to a minimum of 80/40 in hospital.

Investigations.—Robinson-Kepler-Power test: A=8. (Normal: A more than 25.) Thorn test: 30 mg. adrenocorticotropic hormone failed to depress the eosinophil-count appreciably after four hours. Urinary 17-ketosteroid excretion: 1·3 mg. per day (average of two days). Chest radiography: some calcified foci, otherwise no abnormality. Blood chemistry on admission: serum-sodium 137 m.eq., serum-potassium 5·5 m.eq., serum-chloride 92 m.eq., and plasma-bicarbonate 21·4 m.eq. per litre. Blood-urea nitrogen 18 mg. per 100 ml.

Treatment.—On her admission to hospital the patient complained increasingly of a feeling of faintness and she began to vomit. Her blood-

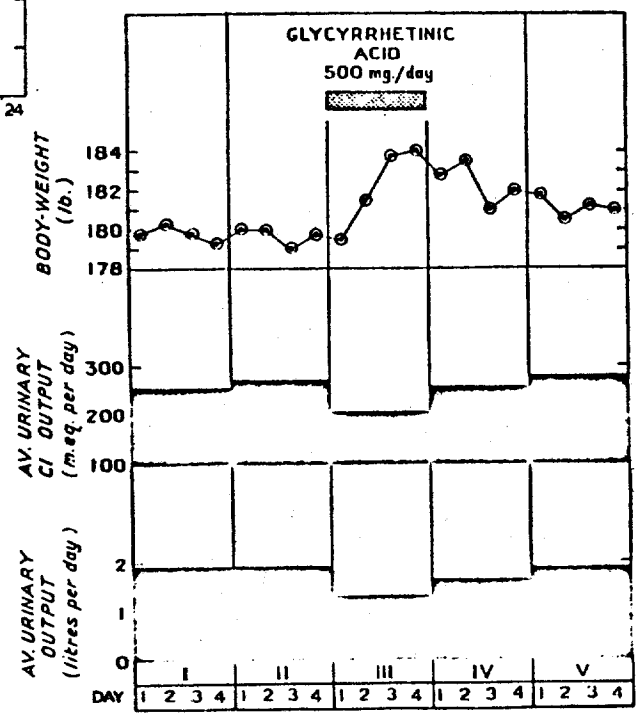

Fig. 3—Effect of glycyrrhetinic acid on daily body-weight, urinary chloride output, and urinary volume in subject C. 4-day periods. Urinary volumes and urinary chloride are expressed as an average for each period.

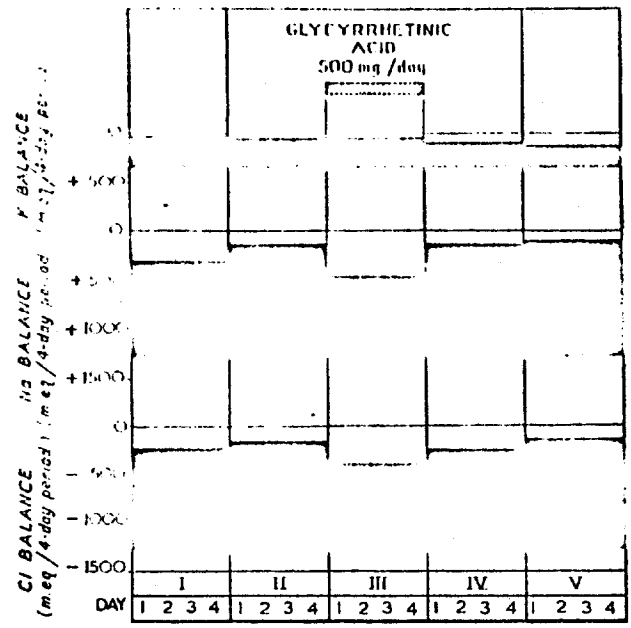

Fig. 4—Effect of glycyrrhetinic acid on potassium, sodium, and chloride balances in subject C. 4-day periods.

pressure had fallen to 90/66. Accordingly it was decided that orthodox treatment should be instituted at once: intramuscular cortisone and deoxycortone were given together and her clinical condition rapidly improved (figs. 5 and 6). Besides subjective improvement, there was a gain in weight of 12 lb. in two weeks. The urine volume diminished, the haemoglobin level fell from 90% (Haldane) to 62%, and the packed-cell volume from 41 to 29%. A metabolic balance study on this patient was thought inadvisable but the changes in the serum electrolytes are seen in fig. 6.

When the patient's condition was satisfactory, active treatment was discontinued and dummy therapy with arachis oil injections and oral lactose was substituted. The body-weight now fell and there was a fall in the serum sodium/potassium ratio, though the general condition of the patient remained good. Oral glycyrrhetinic acid was now substituted for the lactose, and was given in 50 mg. capsules, one q.i.d., for thirteen days. The effects observed (figs. 5 and 6) were similar to those seen with cortisone and deoxycortone therapy. Weight was regained and the daily urinary volume again fell, with concomitant fall in haemoglobin and packed-cell volume. The daily urinary chloride output also fell and the sodium/potassium ratio in the serum increased. On stopping glycyrrhetinic acid there was prompt reversal of these changes.

Apart from the above observations, there was no alteration in the patient's clinical condition, and she was judged well enough to be discharged without maintenance treatment under strict outpatient supervision. That such treatment was in fact necessary was shown by her subsequent admission with a recurrence of symptoms and the characteristic deviation from normal of the serum electrolytes. Conventional therapy was followed by a satisfactory remission.

Conclusion. From this study it appears that glycyrrhetinic acid is capable of reversing certain features of Addison's disease, in a similar way to cortisone and deoxycortone.

EFFECTS ON ANIMALS

These metabolic effects in man have so far not been reproduced in animals. One of us (N. R. W. T.) made the following experiments in rats and guineapigs:

1. Crude liquorice extract was administered by stomach-tube to rats in dosage equivalent to that used in man. In addition, they were given physiological saline solution to drink in order to load them with sodium chloride. The weights of this series of animals were compared with those of an untreated control series, and with a group treated with a small daily dose of deoxycortone (25 µg. per 100 g. body-weight). No increase in weight occurred in the animals treated with liquorice or deoxycortone. Further massive dosage with crude liquorice (equivalent to 180 g. per day for a man of 60 kg.) caused no gain in weight.

2. The same dose of deoxycortone was given to a series of rats which had been unilaterally adrenalectomised and nephrectomised. This group showed no increase in weight as compared with an equal number of similar control rats.

3. Crude liquorice extract was given by mouth to a group of adrenalectomised rats in dosage equivalent to that used in the human experiments. This failed to prolong their survival-time. Likewise, one-third and three times this dose had no effect.

4. Glycyrrhetinic acid was next given to intact rats by mouth and by subcutaneous injection, as well as to adrenalectomised rats. There was no increase in weight in the intact animals and survival-time was not prolonged in the adrenalectomised series. Ten times the equivalent dose used in man also failed in adrenalectomised animals.

5. Glycyrrhetinic acid administered to intact guineapigs by subcutaneous injection caused no increase in weight, either in dosage equivalent to that used in humans, or in doses of ten times this amount.

Conclusion.—From these experiments it would appear that crude liquorice extract and glycyrrhetinic acid have

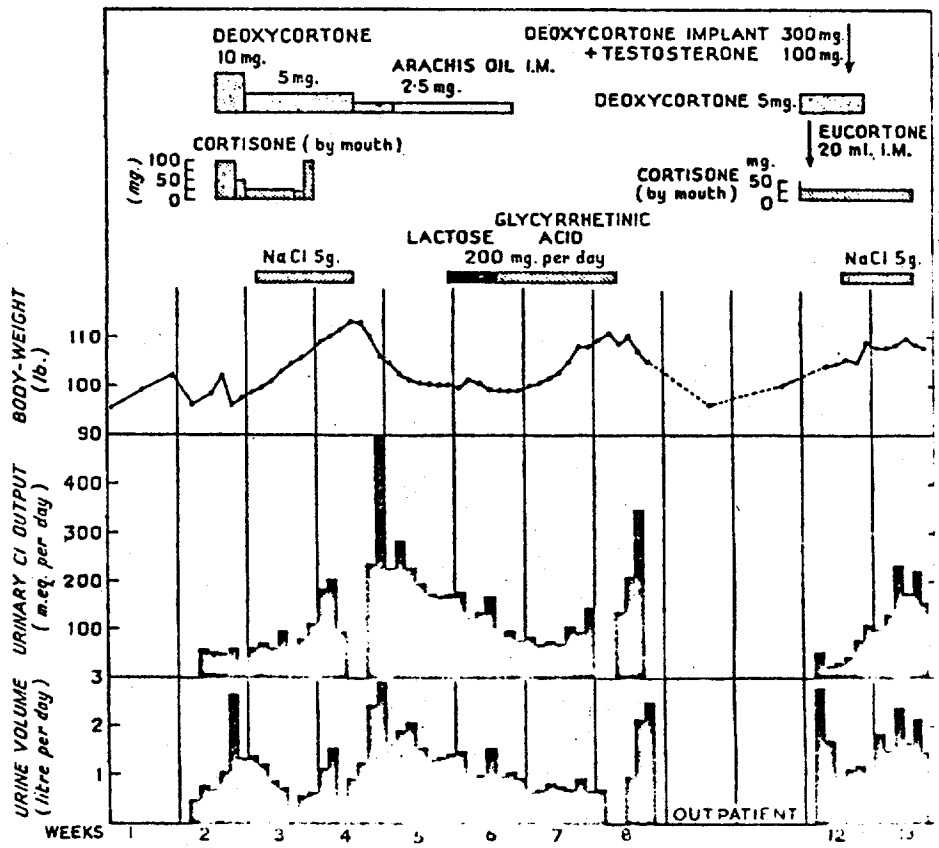

Fig. 5—Daily body-weight, urinary chloride output, and urinary volume in patient with Addison's disease. Weekly periods.

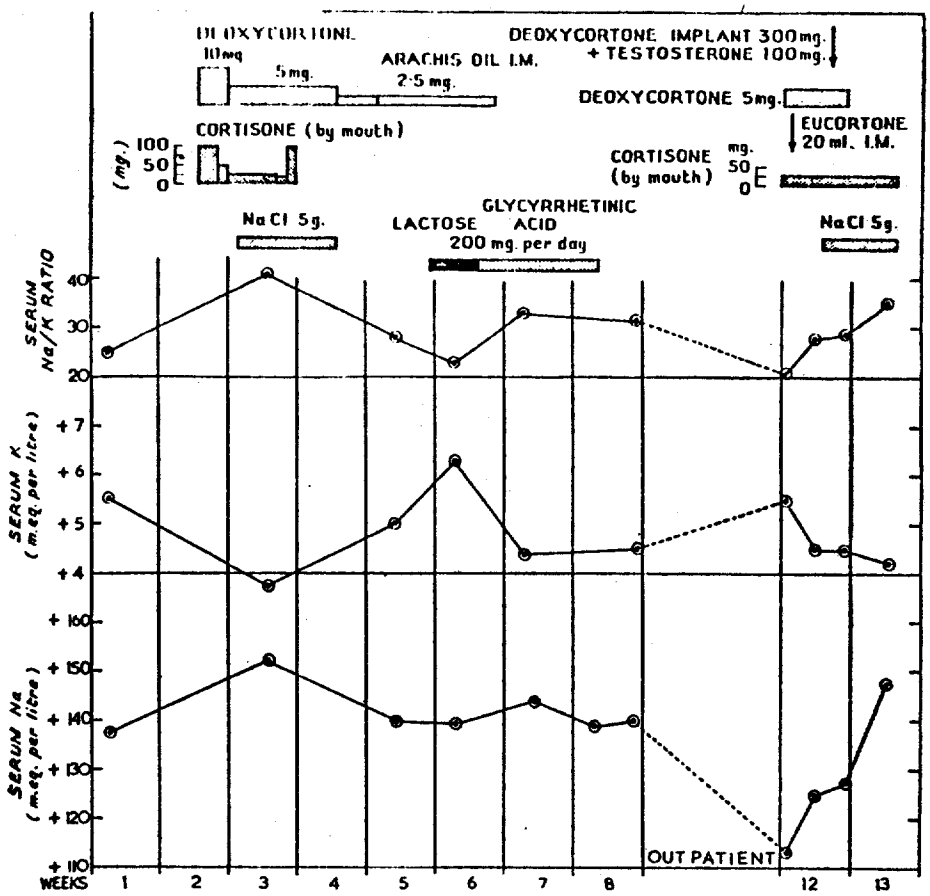

Fig. 6—Serum sodium, potassium, and sodium/potassium ratio in patient with Addison's disease.

no deoxycortone-like action in adrenalectomised rats as judged by survival-time.

DISCUSSION

Previous workers have claimed for liquorice a deoxycortone-like action, with sodium, chloride, and water retention and potassium diuresis. Thus, Molhuysen et al. (1950) present a balance study in a patient with gastric ulcer on succus liquiritiae demonstrating these findings. In our work we confirm the weight gain and sodium and chloride retention on taking liquorice block juice, but we have not observed any striking change in potassium metabolism in normal subjects.

Any attempt to assess the pharmacological activity of liquorice, extracts of liquorice, or purified components will meet several major difficulties. In the first place, the composition of commercial liquorice is very variable. For example, glycyrrhizin content of the raw root is variously stated to lie between 2% and 10%, depending on the country of origin. Secondly, the crude extracts usually imported in this country, such as block juice, are prepared by rough and ready methods, which may well produce considerable variety in the composition of different batches. The "succus liquiritiae" used by Molhuysen et al. (1950) and Groen et al. (1951) is prepared by similar methods. It would therefore be possible to get different effects from "liquorice" as used in different countries.

In addition the variation in individual response to these preparations is considerable. The difficulty in predicting the effect of deoxycortone on water and electrolyte excretion is well recognised (Gaunt et al. 1949). One of our subjects on deoxycortone gained weight, while the other lost weight on a similar dose. Revers (1948) noted that only 20% of his liquorice-treated patients developed œdema and Molhuysen et al. (1950) remarked that none of ten patients treated in hospital with liquorice developed œdema, though it was observed in outpatients.

As previously mentioned, glycyrrhetinic acid was tried on the assumption that it represented the active constituent of liquorice. We are satisfied that this acid reproduced the effects of crude liquorice, and it forms about 50% of glycyrrhizin. The glycyrrhizin content of block juice is variable, but in the specimen we used was probably in the neighbourhood of 10%, that is 20 g. of block juice must have contained about 2 g. of glycyrrhizin. It would therefore seem that glycyrrhetinic acid is perhaps more active than the equivalent weight of liquorice in which it is combined as glucuronide.

If liquorice and its components possess deoxycortone-like activity, one would expect their effects to be most clearly demonstrated in patients with Addison's disease, when the variations in the output of adrenal cortical hormones must be very slight. Molhuysen et al. (1950) used crude liquorice in one patient with Addison's disease without apparent effect, while Groen et al. (1951, 1952) were satisfied that crude liquorice, and ammonium and potassium glycyrrhizinate, were effective in replacing deoxycortone in the control of two patients with Addison's disease. We were able to treat one patient with Addison's disease, and in her the effects of previous and subsequent treatment with deoxycortone and cortisone were reproduced by glycyrrhetinic acid.

We are unable to say how the drug exerts its effect on salt and water metabolism. These effects, however, are most readily explained on the assumption that its action on the kidney is similar to that of adrenal cortical hormones. The excretion of "corticosterone-like" substances (Tompsett 1953) was examined in two subjects during the experiments with block juice. No evidence of increased excretion was noted.

SUMMARY

1. Block juice, a preparation of crude liquorice, is capable of producing water, sodium, and chloride retention in normal individuals.

2. Glycyrrhetinic acid which occurs in liquorice in the form of its glucuronide (glycyrrhizinic acid), reproduced the effects of crude liquorice in normal subjects.

3. Glycyrrhetinic acid, when given to one patient with Addison's disease, had effects on weight and electrolytes similar to those found after the administration of deoxycortone and cortisone.

4. In contrast to deoxycortone, crude liquorice and glycyrrhetinic acid did not prolong the survival of adrenalectomised rats.

We wish to thank Miss A. H. Halley, dietitian at the Western General Hospital, for much help with the preparation of the diets. Messrs. Stafford Allen & Sons (through one of us, W. M.) kindly provided all the preparations of liquorice used in this investigation.

References overleaf

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Human Metabolism of Orally Ingested Glycyrrhetinic Acid and Monoammonium Glycyrrhizinate*

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Triterpene glycyrrhetinic acid (G.A.) and the ammonium salt of its diglucuronide, monoammonium glycyrrhizinate (MAG) have been studied in view of their reported adrenocortical-like activity effects on electrolyte metabolism(1,2,3,4). Other authors found G.A. had potential anti-inflammatory properties(5,6,7) and blocked *in vitro* the metabolism of certain steroids(8). Since there are no known chemical methods of determining G.A. or its metabolites, and since we have previously shown (9) that totally adrenalectomized human subjects, maintained solely on MAG, excreted significant amounts of steroid-like materials in urine, as indicated by formation of Porter Silber (phenylhydrazone) chromogens, we studied the *in vivo* metabolism of G.A. and MAG randomly labelled with tritium.

Methods, apparatus and materials. Both MAG and G.A.‡ were labeled with tritium by the New England Nuclear Corp., following which they were recrystallized to constant specific activity (15.7 μc/mg and 63.6 μc/mg respectively. All countings were done using a Packard Tri-Carb liquid scintillation counter. Complete urine collections from subjects receiving labeled compounds were pooled, concentrated *in vacuo* to about 1/20th original volume, and extracted with ethyl acetate to give the "free" fraction. Urines were then brought to pH 11 and exhaustively extracted with n-butanol to remove the "conjugates." Butanol was removed *in vacuo*, the residue taken up in pH 4.5 acetate buffer and incubated with beta-glucuronidase (Ketodase, Warner-Chilcott), then extraction with ethyl acetate was repeated to remove materials formerly present as glucuronic-acid conjugates. Residual aqueous phase was brought to pH 0.4 with sulfuric acid and allowed to stand 2 days at 37°C to bring about further hydrolysis, and similarly extracted. Radioassay of aliquots of all organic extracts, as well as all aqueous phases was carried out. Fecal samples were extracted twice by homogenizing with alcohol in a Waring Blendor, and filtered. (Soxhlet extraction of dry residue remaining after second hemogenization gave negligible additional activity.) The column chromatographic system used, both on urinary and fecal extracts, was a modification of that described by Van Katwijk and Huis in't Veld (10), in which the crude material is placed on a florisil column in benzene solution and eluted with increasing concentrations of alcohol in benzene. In all cases, when counting aqueous samples (blood and bile) or highly colored extracts of organic solvents (from urine and feces), known amounts of tritium standard were added to the samples after counting to obtain a correction for the quenching caused by color and/or solvent.

Results. Values of urinary excretion of radioactivity following oral administration of labeled MAG and G.A. to 4 human subjects are shown in Table I. Urines of female subjects, subsequently concentrated and used for isolation studies, showed the same degree of activity both before and after concentration, thus eliminating the possibility of large-scale formation of $^3HO^3H$ which would have been lost in the distillate upon concentration. Excretion of a very minute percentage of administered radioactivity in urines was confirmed by results with normal male subjects, and, in addition, presence of practically all radioactivity in feces within 48 hours following ingestion of tagged compounds was conclusively demonstrated. Purification of the fecal extract of the male subject,

* This work supported by grants from U.S.P.H.S., and from G. D. Searle & Co., Chicago, Ill.

† Presented at meeting of Fed. Biol. Soc., 1959, v18, 349.

‡ Part of MAG and G. A. were supplied through kindness of Dr. Willard Hoehn, G. D. Searle & Co.

TABLE I. Metabolic Fate of H^3-Labeled MAG and G.A.

Age	Sex	Diagnosis	Amt. day MAG	Amt. day G.A.	Duration, days	% adm. activity found in Urine	% adm. activity found in Feces
48	♀	Breast cancer – adrenalectomized	*1 mC H^3		5	.3	
50	♀	11 yr Addison's disease		†1 mC H^3	5	.4	
35	♂	Normal	*1 mC H^3		1	.8	98
40	♂	"		†2 mC H^3	1	.4	"

* Plus 4 g carrier. † Plus 3 g carrier.

fed G.A., resulted in isolation of a small quantity of radioactive, crystalline material whose infrared spectrum was the same as that of the administered compound (Fig. 1). This shows that at least a portion of G.A. is excreted unchanged in the feces. A complete characterization of all radioactive fractions has not yet been accomplished.

The next problem was to determine whether MAG and G.A., under these conditions, pass directly through the GI tract without being absorbed (except to a very small degree) or whether they are absorbed into the blood and later excreted via the bile. To answer this question, 5 normal human subjects were fed the labeled compounds and radioassays were performed on simultaneously collected blood and urine samples, and, in 2 of these subjects, on bile. The results (Table II) show that values are all 1% or less of total dose, so that absorption into the blood and re-excretion via the bile may be dismissed as a major metabolic pathway of these ingested materials.

Finally, we were able to isolate and identify by infrared spectroscopy pure glycyrrhetinic acid from the "free" urine fractions of both female subjects, who were fed G.A. and MAG (Table I). Thus it is apparent that the carbohydrate moiety of MAG is hydrolyzed in the body to give the free G.A. as an excretion product. The infrared spectra of these urinary isolation products are the same as those of authentic G.A. and the fecal

TABLE II. Urine, Blood and Bile Levels of H^3-Labeled MAG and G.A.

Age	Sex	Adm. cpd.	% excreted in urine in 4 hr	% found in blood at 4 hr*	% excreted in bile in 4 hr
30	♂	1 mC MAG	.6	.09	
54	♂	*Idem*	.4	.0	
40	♂	"	.3	.2	.0
27	♂	2 mC G.A.	.2	1.2	
70	♀	*Idem*	.05	.4	.15

* Assumed blood vol = 4.2 l and HCT = 46%.

product shown in Fig. 1. These compounds isolated from urine do not, however, comprise the entire amount of radioactive material excreted in urine. Other radioactive "free" fractions are present which show different column chromatographic characteristics, so it is assumed that these represent other compounds

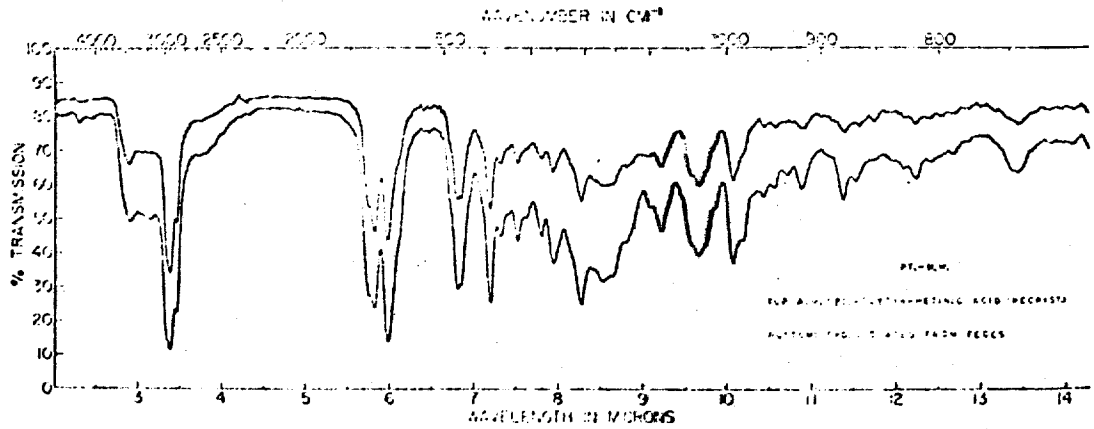

FIG. 1. Infra-red spectra in KBr of glycyrrhetinic acid, administered and isolated from feces.

not yet identified. In addition, identity of the conjugated material is unknown.

Discussion. Throughout the literature dealing with the physiological effects of G.A. and its glucuronide derivative MAG, it was necessary to give large oral doses each day for these effects to become manifest, and parenteral administration has not been feasible in view of their high degree of insolubility in aqueous media. From our results, it seems likely that the necessity for such large oral doses is due to the fact that MAG and G.A., when orally administered, are very poorly absorbed, so that oral doses of 3 to 4 g/day are probably necessary to result in very small circulating levels of parent compounds or their metabolites. Thus the actual circulating "effective amounts" of these compounds or their metabolites may be as small as those of many active steroids. If so, the problem may resolve itself into one of availability rather than one of physiological effectiveness. Variations among several G.A. samples in this regard, as reflected in greater or lesser degrees of absorption, may be related to the stero characteristics of the molecule, as discussed below. However, in view of the wide range of biological activities of MAG and G.A. demonstrated by many researchers, further studies on more efficient modes of administration and or changing the molecule so that it may be more easily absorbed when given orally would seem to be indicated.

Unfortunately, when dealing with G.A. at the present time, one is faced with a lack of definite knowledge regarding the various stereoisomeric forms under which this compound may exist. And, since it can exist theoretically as many isomers, it is possible that these may possess different physiological as well as physical properties. This could explain the contradictory reports in the literature regarding effectiveness of G.A. in performing certain physiological functions, since the investigators often used material commercially sold by various sources as "glycyrrhetinic acid" or "Monoammonium Glycyrrhizinate," and failed to characterize their compounds physically. Thus the G.A. used in one laboratory may not have been at all the same as that used in another.

The G.A. used by us is likewise of uncertain stereoisomeric structure. Its infrared spectrum is identical with that of a commercial sample designated as beta G.A.§ as well as with that of an experimental sample of 18-beta G.A.‖ On the other hand, the specific rotation of our material is lower than that of the other 2 samples by 50 degrees, and its solubility in alcohol and chloroform is less. It is not 18-alpha G.A., since it is different in all properties, including infrared absorption, from a commercial sample of alpha G.A.§, which is probably the same as the 18-alpha compound of Beaton and Spring(11). Our compound has been purified by recrystallization, as well as by charcoal treatment of the Na salt, followed by precipitation of the free acid, and we have been unable to produce any significant change in either its infrared spectrum or specific rotation. The same procedures were carried out on both the commercial alpha and beta samples, again without change of characteristics. It may therefore be that our compound is beta oriented at the same position as are the others, but differs from them in some other position which does not cause a change in absorbance of infrared light but which affects the specific optical rotation. What this position might be we do not know.

Summary. Orally administered glycyrrhetinic acid and monoammonium glycyrrhizinate were poorly absorbed from the gastro-intestinal tract as indicated by blood, bile and urine levels of radioactivity. The bulk of fed materials are excreted directly in the feces, and, in the case of G.A., at least in part is unchanged compound. Small quantities of glycyrrhetinic acid have been isolated from urines of subjects fed each of above compounds. Many problems relating to stereoisomerism of G.A. remain to be clarified.

§ Generously supplied by Dr. R. R. Blumenthal, S. B. Penick & Co.

‖ Kindly donated by Dr. F. S. Spring, Royal Technical College, Glasgow, Scotland.

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Licorice Poisoning, Pseudoaldosteronism, and Heart Failure

To the Editor.—The curious syndrome of pseudoaldosteronism caused by excessive licorice ingestion usually causes muscle weakness, hypertension, and paresis. The following case is unusual in that a previously healthy patient presented in fulminant congestive heart failure (CHF) after ingesting large quantities of licorice for one week.

Report of a Case.—A 53-year-old man suffered shortness of breath, ankle edema, increasing abdominal girth, weight gain, headache, and weakness for one week. On the eve of his admission he sat in a chair all night, unable to sleep lying down. He had been in excellent health, was taking no medications, and had never been previously hospitalized. He had always been a heavy salt and water user. He had eaten 700 gm of licorice candy beginning nine days earlier, having consumed the last piece one day prior to admission.

Physical examination revealed a well-developed man in moderate respiratory distress. The neck veins were distended to the angle of the jaw at 90° with a positive hepatojugular reflex. Many rales were heard at both lung bases with some dullness to percussion on the right. The point of maximum intensity was 1 cm outside the midclavicular line in the sixth interspace and third and fourth heart sounds were heard. There were no murmurs. The liver was palpable 6 cm below the costal margin and percussed to 14 cm. A fluid wave and shifting dullness were present. Moderate pitting edema of both legs was evident.

The blood pressure was 160/90 mm Hg. Laboratory tests revealed the following values: serum sodium 144 mEq/ml, potassium, 3.4 mEq/ml; chloride, 105 mEq/ml; carbon dioxide 28 mEq/ml; venous pH, 7.46; creatinine clearance, 91.5 ml/min; 17-ketosteroids, 15.4 mg/24 hr, and 17-hydroxycorticosteroids, 22.6 mg/24 hr. A 24-hr urinary aldosterone level determination was 5.4μg/24 hr (5μg to 20μg/24 hr), and an ambulatory plasma renin level was below detectable limits (less than 30 ng/100cc). An electrocardiogram (ECG) showed flattened T waves and prominant U waves suggestive of hypokalemia, but was otherwise normal. An x-ray film of the chest (Figure) showed the heart size to be at the upper limits of normal with pulmonary vascular congestion and a right basilar pleural effusion.

With a 2-gm sodium diet and bed rest, but no medications, the patient lost 12 lb over the next four days by diuresis. Needless to say, further licorice was withheld. He became asymptomatic, the x-ray film of the chest improved, the T-wave changes on the ECG became normal, and the blood pressure returned to a normal of 128/80 mm Hg. Four days after admission he was discharged, to receive spironolactone, 25 mg three times a day for one week. Three weeks after discharged, all studies gave normal results. The x-ray film of the chest had totally cleared, the sodium level was 138 mEq/ml and the potassium level was 4.4 mEq/ml. Although he subsequently returned to his high salt and water intake, he is still in good health one year after admission.

Comment. The active principle in licorice extract has been shown to be glycyrrhizic acid, a substance structurally and chemically similar to desoxycorticosterone and aldosterone.[1,2] Revers,[4] reporting on the effective treatment of ulcers with licorice extract, was the first to describe its side effects which included "cardiac asthma or edema" in 20% of his patients. In a search of the world literature, two other patients were found who developed CHF while receiving high doses of licorice. Both were being treated for Addison's disease and were on a high daily sodium intake.[5,6]

Congestion and right pleural effusion.

The signs and symptoms of licorice-induced pseudoaldosteronism have been well summarized by Conn et al,[7] and include hypertension, hypokalemic alkalosis, suppressed aldosterone and renin levels, myoglobinuria, paresis, tetany, edema, headache, and weakness. The patient reported herein had most of the above signs and symptoms with the notable exception of tetany, paresis, and myoglobinuria which would be expected in those with a more chronic licorice ingestion resulting in marked hypokalemia.

Since the complication of CHF secondary to licorice ingestion is rarely suspected or sought for, its true incidence is unknown. If this previously healthy patient could develop such severe CHF and pulmonary edema from licorice it would even more likely affect someone with pre-existing cardiac compromise. Although some form of cardiomyopathy could have existed in this patient, it would seem unlikely in view of his normal exercise tolerance, ECG, and treadmill exercise test. Licorice is probably consumed in higher quantities in the European countries. Nevertheless, it is certainly ubiquitous enough in America and probably unsuspectingly complicates treatment in many patients.

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William A. Hines, MD, gave permission to study his patient.

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GLYCYRRHIZINOPHILIA AS A CAUSE OF EDEMA

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In an increasing majority of patients with edema, the cause of the edema can be delineated. Congestive heart failure, renal disease and diseases associated with hypoalbuminemia constitute the large proportion of these causes. Local interference with venous or lymphatic return often produces localized edema. Increase in capillary permeability may be a factor, particularly in those situations associated with inflammation.

More recently, Conn[1] has reported a syndrome which may be accompanied by edema and is due to increased production of aldosterone with accompanying sodium retention. That many types of adrenal hyperfunction may be associated with edema is well recognized as is the edema produced by the administration of certain steroids.

Despite the clear understanding of these many causes, a small number of people have edema due to seemingly inexplicable causes. Milroy's disease (perhaps due to a congenital lack of lymphatics) stands among these few.

The patient herewith reported seemingly had inexplicable edema until the true cause was discovered. With time and understanding, more and more "*idiopathic*" causes of edema will be clearly understood.

CASE REPORT

A 19-year-old woman was first seen on September 14, 1960. For the past year she had noticed chronic edema of the legs and ankles. There was no history of thrombophlebitis. She had had repeated urinalyses, all of which had been negative. There was no prior history of heart disease. Repeated cardiac examinations since the onset of her edema had been negative.

On physical examination her blood pressure was 180/100. The neck veins were normally full without distention. There were no rales at the lung bases and there was no hepatomegaly. The skin was of normal texture and there was no lymphadenopathy. There was a 2+ pitting edema of the lower extremities.

A complete urinalysis revealed no albumin, no pus cells or casts and a specific gravity of 1.016. A 24-hour urine was also negative for albumin. The total serum protein was 6.6 gm. percent with an albumin of 4.2 gm. percent and a globulin of 2.4 percent. An x-ray of the chest was negative. The heart was of normal size and contour. The packed cell volume was 41 percent, the white blood count 11,333 and the differential showed 8 percent lymphocytes, 1 percent monocytes, 81 percent segmented cells, 7 percent band cells, 1 percent eosinophiles and 2 percent basophils. A bromsulphalein liver function test showed 26.3 percent retention in 45 minutes. The blood urea nitrogen was 5 mg. percent. An x-ray of the lumbosacral spine was negative with normal renal and psoas outlines. A Papanicolaou smear of the cervix was negative. A circulation time was 13 seconds (arm to tongue) and venous pressure, 50 mm. saline. The thymol turbidity was 2 units and an alkaline phosphatase was 6.5 units. The serum sodium was 140 mEq. and the serum potassium 4.4 mEq.

At the time she was seen this woman weighed 141 pounds. There was no apparent cause of her edema. After several discussions with her regarding the possible etiologic factors, by coincidence, it was discovered that she was eating large amounts of licorice. She would keep a box of licorice candies in her desk and nibble on them through the afternoon. In addition, after detailed questioning, the fact was elicited that on the day prior to the onset of her edema she had eaten 35 cents worth of licorice while watching a show. When the detailed questioning about the licorice began, the patient herself completely stopped eating it. By the time she returned five days later, she had lost 4 pounds. It was then that the serum sodium and potassium were obtained. With continued abstinence from licorice, she progressively lost weight from 141 pounds to 125 pounds. She has remained free of edema with a consistent weight of 125-128 pounds, except on one occasion when she again become edematous. She recalled having eaten some candies the previous day and on inspection they were found to have licorice centers. With abstinence from the candies, there was a diuresis 24 hours later and there since has been no recurrence of the edema.

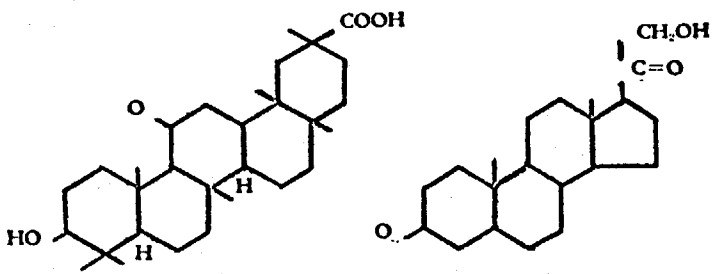
DISCUSSION

In 1946, Revers noted that while patients with peptic ulcer benefitted

symptomatically from the ingestion of licorice, they occasionally developed edema.[2] Subsequently, the observation of the development of edema with ingestion of licorice has been made repeatedly.[3] The production of a syndrome simulating that described by Conn due to hypersecretion of aldosterone has been described.[4]

The active principle of licorice has been separated as glycyrrhizinic acid and has been found to bear some resemblance to the adrenal steroids in its structure (Table 1).

TABLE 1



Glycyrrhizic acid 11-Desoxycorticosterone

The action of this material has been studied by Louis and Conn[5] and others. In the form of ammonium salt it does produce definite sodium retention and potassium diuresis. The sodium retention is accompanied by fluid retention and weight gain. There is reduction of sodium and chloride in sweat. There is a definite fall in urinary 17-ketosteroids.

The ingestion of this compound has been shown to produce renal arterial disease and hypertension in animals.[6]

A patient with Addison's disease with glycyrrhizinophilia has been reported. Beneficial effects of this compound in Addison's disease[7] has been noted.

Although no conclusive studies have been accomplished on the patient, particularly studies of adrenal function while off and on the drug, we believe there is reasonable evidence that she had developed edema as a result of licorice ingestion. She was eating large amounts of the material every day. She had no other demonstrable cause of edema. The cessation

of licorice ingestion was followed by a definite diuresis with a weight loss of 15 pounds.

SUMMARY

Glycyrrhizinic acid, the active component of licorice, has a sodium-retaining, aldosterone-like effect. Ingested in excessive amounts, it is capable of producing the features of hyperaldosteronism.

a. Sodium retention

b. Potassium diuresis

c. Hypertension

A case of edema probably due to excessive ingestion of glycyrrhizin is reported.

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THE ACTION OF MONO-AMMONIUM GLYCYRRHIZINATE ON ADRENALECTOMIZED SUBJECTS AND ITS SYNERGISM WITH HYDROCORTISONE*

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MOLHUYSEN *et al.* (1) demonstrated that licorice extract taken orally by human subjects acted on the electrolyte balance in a manner similar to that of desoxycorticosterone acetate, namely, with the retention of sodium and the excretion of potassium. Groen and his associates (2, 3, 4) extended these findings to demonstrate that glycyrrhizinic acid[1] and its hydrolysis product, glycyrrhetic acid obtained from licorice, were the active ingredients of the extract. Though Groen *et al.* had some success in the maintenance of patients with Addison's disease on licorice preparations, Borst and his associates (5) noted that these preparations in a case of Addison's were ineffective when used alone. However, they did note that small doses of cortisone used in conjunction with licorice were quite effective in maintaining the electrolyte balance in adrenal deficiency states, and suggested that those cases of Addison's disease which did respond to licorice still had remnants of functional adrenocortical tissue (5). Recently Hudson *et al.* reported some studies on the use of small doses of cortisone with licorice preparations in adrenalectomized patients (6, 7).

This study[2] comprises data on the effectiveness of mono-ammonium glycyrrhizinate (MAG) in the maintenance of 2 adrenalectomized patients. Clinical and metabolic observations were made during a period in which varying doses of MAG and hydrocortisone were administered. Laboratory observations included determinations of serum potassium, serum sodium and plasma cholesterol levels, blood eosinophil counts, and urinary sodium, potassium and 17-ketosteroid excretion.

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* This investigation has been aided by grants from MacAndrews Forbes Company, Camden, New Jersey, and the U. S. Public Health Service, No. M657.

[1] Glycyrrhizinic acid consists of a disaccharide moiety and one molecule of the triterpine, glycyrrhetinic acid.

[2] The authors are deeply indebted to Dr. Lincoln Lebeaux for his invaluable assistance in the execution of this study.

Mono-ammonium glycyrrhizinate was kindly supplied by Dr. C. K. Swift of MacAndrews Forbes Company.

ADRENALECTOMIZED SUBJECTS

The subjects selected for this study were 2 chronic schizophrenic female patients (C. P. and P.M.), in each of whom the psychosis was of fifteen years' duration. Both patients had had carcinoma of the breast for which radical mastectomy had been performed prior to their admission to Worcester State Hospital. Bilateral adrenalectomy was performed on June 16, 1952. Although both patients were said to have metastasis, evidence of metastasis was observed only in Patient C.P., who prior to adrenalectomy had an indurated immobile mass the size of a lemon in the postoperative mastectomy scar on the left. Following the adrenalectomy, the mass gradually decreased in size, and by the time of this study was no longer palpable.

In preparation for the mono-ammonium glycyrrhizinate (MAG) experiment, the daily oral dose of 80 mg. of hydrocortisone which the patients were receiving while other studies were in progress, was gradually reduced over a period of sixteen days to 40 mg. per day, at which time the MAG was started in a dosage of 4.0 Gm. by mouth daily with 2.0 Gm. of sodium chloride. *The sodium chloride (2.0 Gm. per day) was given throughout the entire study.* Prior to the beginning of the experiment, both patients were alert, active and voiced no somatic complaints. Both patients had voracious appetities and slept well. During the sixteen-day period immediately prior to the administration of MAG, the blood pressure of Patient C.P. ranged from 144/70 to 130/80, and that of Patient P.M. varied between 104/56 and 102/70.

CASE HISTORIES

Subject C.P.

On May 4, 1953, MAG was started in a dosage of 4.0 Gm. a day orally, in conjunction with oral hydrocortisone, 40 mg. daily. The patient was kept on this regimen for seven days. The medication schedule may be seen in Figure 1. During this period no changes were observed, except a tendency to sleep more. On May 11, the hydrocortisone was reduced to 20 mg. a day. During this period the patient's appetite, though adequate, was not voracious as it had been and she was less active. On May 13, pitting peripheral edema was noted for the first time and this persisted for three days before subsiding. During one period when MAG was reduced from 4.0 to 2.0 Gm. daily (May 18–22), the patient's appetite became poor, she was lethargic and exertional dyspnea was noted for the first time. When MAG was increased to 4.0 Gm. a day, the patient showed marked clinical improvement and despite the fact that on May 25 the hydrocortisone was reduced to 10 mg. per day, her condition remained satisfactory for the next eleven days. The lethargy disappeared and her food intake returned to its previous ravenous level. The only abnormality on physical examination during this period was an occasional extra cardiac systole.

On June 8, hydrocortisone was discontinued and the patient was maintained on MAG, 4.0 Gm. per day. For the first twenty-four hours, no untoward symptoms were

noted. However, by the second day she lost all spontaneous volubility, answered questions with a paucity of words and spoke only in monosyllables. The tendency to sleep became prominent and the patient remained in bed all day. Peripheral edema returned and the blood pressure dropped to 122/72, from the 140/90 observed on June 8. On June 11, the patient was lethargic, weak, anorexic and took small amounts of liquid only after strong urging. There was a marked oliguria and she passed three watery stools during the day. The blood pressure dropped to 114/89, the temperature rose to 100.6° F rectally and the pulse was rapid at an average rate of 104 per minute. Since the patient's condition was becoming worse and circulatory embarrassment was evident, hydrocortisone was resumed in a dosage of 10 mg. per day orally. On June 12, a clysis of 1,000 cc. of 5 per cent glucose was given. No improvement was evident for the next thirty-six hours. However, by the evening of the second day after resumption of hydrocortisone, definite improvement was discernible and by the third day the patient was eating well, spoke spontaneously and got out of bed. By June 17, the peripheral edema had disappeared and no signs of circulatory embarrassment were evident.

On June 29, hydrocortisone was again discontinued and the patient was maintained on MAG, 4.0 Gm. per day. The clinical status was characterized by increasing irritability, restlessness, lethargy, lack of appetite, weakness and somnolence—a clinical picture which was similar to that which occurred during the previous period when the hydrocortisone was discontinued. On July 4, oral hydrocortisone in a dosage of 20 mg. per day was begun. The patient's condition was again markedly improved. Gradually the hydrocortisone was increased to 40 mg. a day and on July 15 the MAG was discontinued. The clinical status of the patient was satisfactory during the remainder of the study.

Subject P.M.

In Patient P.M. the medication schedule was similar to that in Patient C.P. On May 4, 1953, MAG was started in a dosage of 4.0 Gm. daily, the oral dose of hydrocortisone having been gradually decreased over the preceding sixteen days from 80 mg. per day to 40 mg. per day. The following day the patient became restless, paced the floor and although she frequently returned to bed, did not sleep soundly. The next day her face was swollen and an erythematous flush of butterfly configuration covered the nose and the malar prominences. This condition was diagnosed as erysipelas. Although the patient's temperature was not elevated, she was given penicillin, 300,000 units a day, for three days. By May 8, the swelling and discoloration of the face had subsided. She recovered from this facial infection with no signs of adrenal deficiency; there was no increase in the daily dose of hydrocortisone. When the dosage of hydrocortisone was reduced to 20 mg. a day on May 11, pitting peripheral edema developed; she complained of fatigue and was quite lethargic. These untoward manifestations persisted for two days and then spontaneously disappeared without change in the medication schedule. On May 18, the hydrocortisone was decreased to 10 mg. and the MAG to 2.0 Gm. daily. Despite the development of a florid perineal cellulitis which required 600,000 units of penicillin for relief, the patient's general physical condition remained satisfactory and there were no signs of adrenal embarrassment. On May 22 MAG was increased to 4.0 Gm. daily.

On June 8, the hydrocortisone was discontinued, but the MAG was kept at 4.0 Gm. per day. At 6:00 A.M. the following day the patient complained of nausea and vomited a small amount of greenish liquid. The temperature was 100.4° F and the pulse was weak and rapid at 114 per minute. The blood pressure was stable at 120/80. The following day

the temperature rose to 102° F. Because of the hyperpyrexia, persistent nausea and vomiting, tenderness to palpation in the right upper quadrant and the history of a previous attack of empyema of the gallbladder, a diagnosis of acute cholecystitis was made. On June 10, penicillin was started in a dosage of 600,000 units twice a day, and parenteral fluids were given.

On June 12, the patient had a brief period of unconsciousness during which she slumped in the nurse's arms while attempting to use the bed pan. The blood pressure dropped to 100/80, and the possibility of impending adrenal crisis prompted the resumption of hydrocortisone, 10 mg. per day. The patient responded well to hydrocortisone at this dosage level and a satisfactory clinical status was restored on June 14. Throughout the next two weeks her condition was good, with the maintenance of normal eating and sleeping habits.

On June 29, hydrocortisone was again discontinued. On July 1, the patient was observed to be drowsy. Drowsiness continued during the following day, but physical examination revealed no other abnormality. On July 3, she was lethargic, complained of nausea, and refused to eat dinner or supper. During the night she retained only small amounts of fluid. However, no circulatory embarrassment was evident and the blood pressure was 112/80. On July 4, hydrocortisone was resumed in a dosage of 20 mg. a day orally. The patient remained listless and nauseated, but did not vomit. On July 7, she had pain in her left shoulder and vomited periodically throughout the day. She complained of feeling cold and her skin was cold to touch, although her temperature was 99.8° F rectally. Her blood pressure dropped to 90/52, the pulse was feeble and rapid, and circulatory embarrassment was evident. The inability of the patient to retain oral medication necessitated the intravenous administration of hydrocortisone, 100 mg. in 500 cc. of saline. Though a favorable clinical response to this measure was promptly discernible, it was of short duration. The following day she again lapsed into a state of crisis. She was given hydrocortisone, 100 mg. in 500 cc. of saline intravenously, and cortisone acetate, 25 mg. three times a day intramuscularly. She responded favorably to these measures and by the following evening her clinical status had significantly improved. This improvement was maintained. On July 12, the cortisone acetate was reduced to 25 mg. per day intramuscularly and on July 16 it was discontinued. On July 17 the MAG was discontinued and the patient remained in good clinical condition for the remainder of the study on an oral maintenance dosage of 40 mg. of hydrocortisone daily.

Both of these patients had had schizophrenia for many years, for which they had been institutionalized. At the time this study was instituted both patients showed the cardinal signs of this illness, *i.e.*, a flat, inappropriate affective response, disorganization of thought processes, tangential and irrelevant associations, restriction of the scope of awareness and a complete loss of insight. The psychiatric status of both patients remained unchanged throughout the entire period of study, except on those occasions when they lapsed into an adrenal crisis. On such occasions both patients showed none of the cardinal signs of schizophrenia on examination. However, as soon as adrenal balance was restored by appropriate treatment the unmistakable signs of an active schizophrenic process again became evident in both subjects.

METABOLIC STUDIES

Blood

Figures 1 and 2 show the blood pressure, eosinophil counts, serum Na/K ratio, plasma cholesterol level, and the medication schedule of MAG and adrenal steroids for C.P. and P.M., respectively.

In general the eosinophil pattern was similar in both patients. During the first period of hydrocortisone discontinuance an abrupt increase in the count was noted. With return to a dosage of 10 mg. of hydrocortisone a day, the count became stabilized at the higher levels. Again, with the second

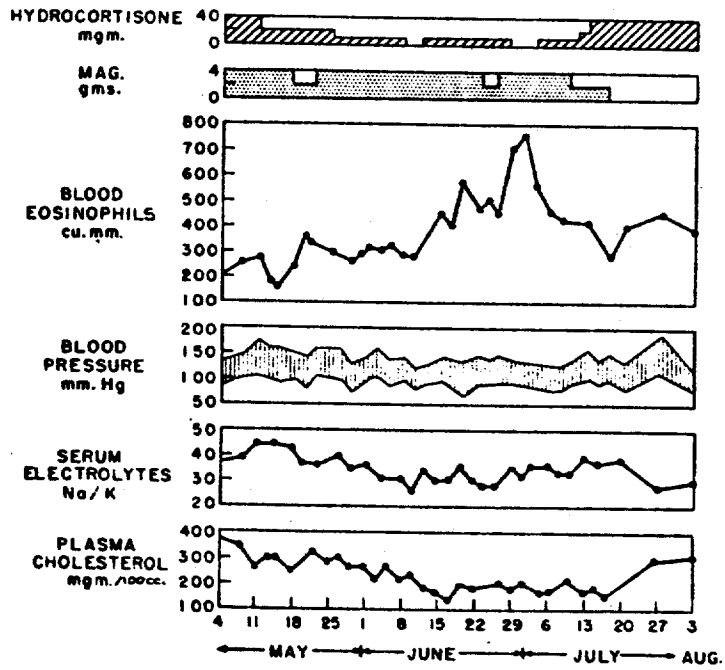

FIG. 1. Medication schedule and blood data on adrenalectomized Patient C.P.

period of steroid abstinence the eosinophil count rose promptly. It may be observed that Patient P.M. showed a pronounced rise in the eosinophil count during the adrenal crisis; following the intravenous administration of 100 mg. of hydrocortisone there was a marked drop. By the end of the study period the eosinophil counts had decreased steadily. However, both patients at this time showed higher eosinophil counts than at the initiation of the observations.

There is evidence to suggest that the serum Na/K (molar ratio) and blood pressure are positively related. Increases and decreases of the Na/K ratio followed very closely the corresponding changes in blood pressure. In the course of the study it became evident that these fluctuations were also

related to the clinical condition of the patients (*see* case histories). The blood pressure and electrolyte ratio at the end of the study, when a dose of only 40 mg. of hydrocortisone was being administered, were lower than when 40 mg. of hydrocortisone was supplemented by 4.0 Gm. of MAG at the beginning of the observation period.

A feature of the blood chemistry data which was not related to the clinical condition of the patients but rather to the dose of hydrocortisone, was the plasma cholesterol level. In both patients a steady decline in the

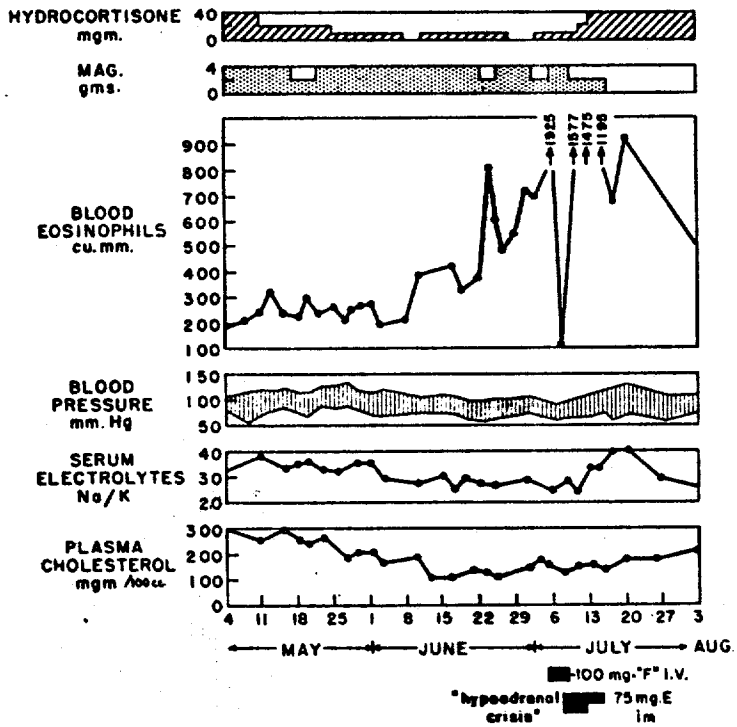

FIG. 2. Medication schedule and blood data on adrenalectomized Patient P.M.

cholesterol values was associated with a reduction in the dose of hydrocortisone. The lowest cholesterol values were observed during the periods when the patients received no hydrocortisone or when the dosage was reduced to 10 mg. a day. With the return to higher dosage levels of hydrocortisone there occurred a steady rise in the cholesterol values to those observed at the initiation of the study.

Urine

Urine collections were made on both patients during the study. Patient P.M. was more cooperative, and therefore her data are more complete.

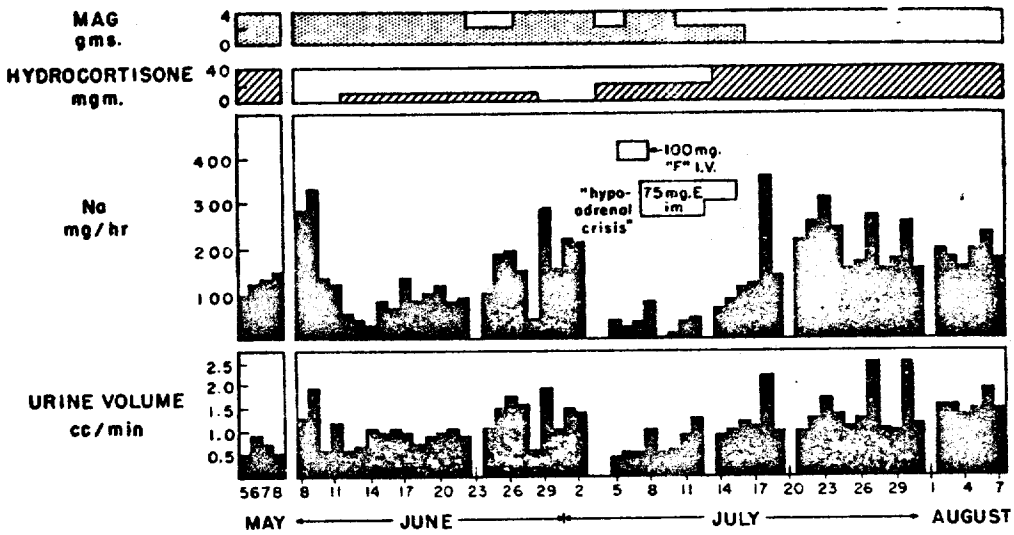

FIG. 3. Rate of urinary sodium and urine excretion in adrenalectomized Patient P.M.

Figure 3 shows the urinary sodium excretion of Patient P.M. recorded in mg. per hour and urinary volume in cc. per minute; and Figure 4 depicts the K/Na ratio (by weight) and the 17-ketosteroid excretion in mg. per twenty-four hours. The corresponding data on Patient C.P. are listed in Table 1.

In Figures 3 and 4 it should be noted that there is a period from May 8

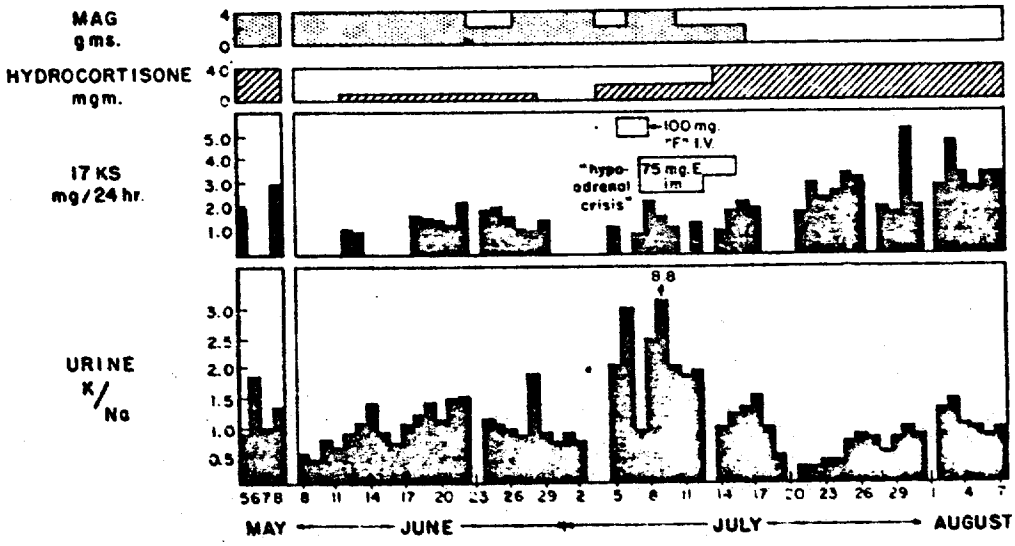

FIG. 4. 17-Ketosteroid excretion and K/Na ratio of urine in adrenalectomized Patient P.M.

TABLE 1. URINARY ELECTROLYTE AND 17-KETOSTEROID EXCRETION OF PATIENT C.P.*

Date (1953)	Urine vol. (cc./min.)	Na (mg./hr.)	K (mg./hr.)	17-KS (24 hrs.)
May 5	.49	120	101	4.32
6	.85	142	153	—
7	.60	115	94	—
8	.69	131	118	2.40
June 8	.49	103	131	—
9	.48	63	35	—
14	.67	18	14	.60
15	.47	33	17	—
16	.52	69	47	—
19	.34	36	48	.62
24	.79	74	41	.70
26	.61	91	36	.53
27	.40	62	37	.72
28	.53	43	26	.72
29	.31	31	44	.76
30	.35	34	26	—
July 7	.43	23	160?	1.82
8	.38	46	53	.87
9	.86	86	103	.98
10	.53	35	30	.65
11	.67	75	99	.86
12	.80	95	110	1.18
14	.81	78	92	1.10
15	.74	90	61	1.20
17	.80	112	72	1.68
18	.50	68	49	1.61
21	.36	74	34	1.20
22	.55	85	45	1.08
24	.80	136	61	1.56
25	.72	212	143	2.38
29	.58	85	102	1.99
30	.74	—	—	2.40
Aug. 4	.76	142	107	2.88
5	1.10	169	111	3.12
6	.94	150	113	2.40
7	.93	164	124	1.80

* *See* Figure 1 for medication schedule, and text for case history.

to June 8 when no collections were obtained.

During the period when Patient P.M. was receiving 40 mg. of hydrocortisone daily supplemented by 4.0 Gm. of MAG, the 17-ketosteroid excretion rate was 2–3 mg. per twenty-four hours. Immediately following the first period, during which hydrocortisone was withheld, the 17-ketosteroid excretion decreased to less than 1 mg. per day. At a dosage level of 10 mg. of hydrocortisone daily the steroid excretion rose slightly, varying between 1 and 2 mg. per day. Again, during the second period of hydrocortisone abstinence beginning on May 29, the steroid excretion dropped to less than 1 mg. per day. With the resumption of larger doses of hydrocortisone an increase in 17-ketosteroid excretion was noted. At the end of the study the excretion values had returned to the levels of 2–3 mg. per day and higher which prevailed at the initiation of the study. It should be noted that during the period of administration of intramuscular cortisone acetate and intravenous hydrocortisone, the increase in 17-ketosteroid excretion was not commensurate with the amount of steroids administered.

The volume of urine excreted appeared to be correlated with the amount of sodium excreted. This was strikingly evident in Patient P.M. (Fig. 3). During the first period in which hydrocortisone was withheld there was a pronounced loss of water and sodium, which was followed by a reduced urinary volume and sodium excretion. During the period of marked clinical deterioration (June 10 through June 11) this relationship was also reflected by a decline in the urinary K/Na ratio (*cf.* Fig. 4). With the resumption of hydrocortisone in a dosage of 10 mg. per day, the sodium excretion slowly increased and continued at the levels which existed a month earlier during the period when the patient was receiving hydrocortisone, 40 mg. daily supplemented by 4 Gm. of MAG. On May 23, when the MAG was reduced to 2 Gm. per day, an increase in sodium excretion was again noted, but this was not as large as during the omission of hydrocortisone. Upon the resumption of MAG (4.0 Gm. a day) on May 27, marked sodium retention was evident by the second day. These changes in electrolyte balance were reflected by decreases and increases in the urinary K/Na ratio. When the patient was maintained on 4.0 Gm. of MAG per day in the absence of hydrocortisone, inability of the patient to retain sodium was again observed. Upon resumption of hydrocortisone (20 mg. per day), the electrolyte pattern returned to normal, showing sodium retention and an increase in the K/Na ratio. However, when the patient was unable to retain oral medication, adrenal crisis occurred. The measures taken to control the adrenal crisis, namely, the administration of hydrocortisone intravenously and cortisone acetate intramuscularly, caused further reten-

tion of sodium and excretion of potassium as indicated by the high urinary K/Na ratio. When the MAG was discontinued on July 17, there was a marked increase in the excretion of sodium, which remained high for the remainder of the study except for a slight decrease observed by the end of the period of observation.

Figure 5 contains data regarding the sodium excretion and urinary K/Na ratio of Patient P.M. during the period in which hydrocortisone (40 mg. per day) was being administered. Placebos of lactose were given during the control period and during the experimental period when the

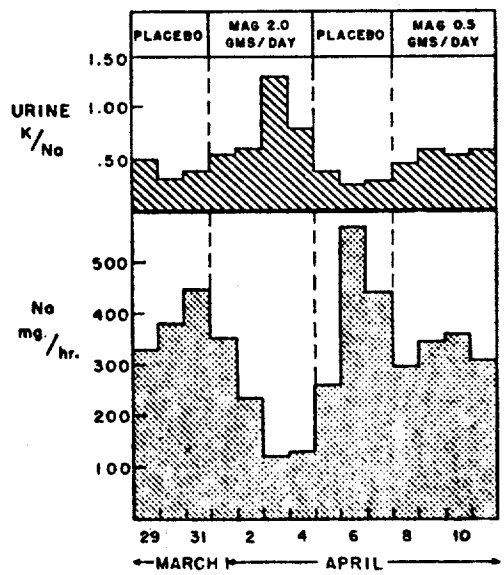

FIG. 5. The effect of varying doses of MAG on the electrolyte excretion of Patient P.M. maintained on 40 mg. of hydrocortisone and 2.0 Gm. of sodium chloride orally per day.

patient was receiving MAG, 2.0 and 0.5 Gm. per day respectively. It may be seen that a daily oral dose of 2 Gm. of MAG has a potent sodium-retaining effect when supplemented by 40 mg. of hydrocortisone, but not when employed in conjunction with a daily dose of only 10 mg. of hydrocortisone (cf. Fig. 3). The smaller dose of 0.5 Gm. of MAG has little if any sodium-retaining effect.

DISCUSSION

MAG alone, given in the dosage employed in these studies, was ineffective in the maintenance of 2 adrenalectomized patients. On two occasions an attempt was made to maintain the patients on 4.0 Gm. of MAG per day alone (2.0 Gm. of salt per day was given throughout the study).

Within the first twenty-four hours there was a loss of sodium and fluid, followed promptly by a decline in the clinical status of both patients which was evident by the second day. However, as little as 10 mg. of hydrocortisone orally in conjunction with the 4.0 Gm. of MAG per day was effective in maintaining a satisfactory clinical state, as well as fluid and electrolyte balance. On the occasions when the dose of MAG was reduced from 4.0 to 2.0 Gm. daily while hydrocortisone was maintained at a level of 10 mg. daily, there was a perceptible decline in the clinical status associated with sodium and fluid depletion. When Patient P.M. was given 2.0 Gm. of MAG while receiving 40 mg. of hydrocortisone per day, there was a sharp reduction in urinary sodium excretion, which was promptly reflected by a corresponding rise in K/Na ratio (Fig. 5).

The results of this study do not supply conclusive answers to the question of whether MAG, when employed alone, is in fact sufficient to maintain life in adrenalectomized patients. Hudson and his associates (6, 7) apparently were able to maintain life in 1 of 3 adrenalectomized patients for several weeks, employing glycyrrhizin alone. However, all 3 patients were in a grave clinical condition throughout the period of treatment. The occurrence of drowsiness and lethargy during the abstinence from hydrocortisone is not, in all probability, due to a specific MAG effect, but rather to the state of extreme hypoadrenalism. These studies confirm the observation of Borst *et al.* (5) to the effect that MAG alone is ineffective in the maintenance of certain patients with Addison's disease in whom there are no remnants of adrenal tissue.

The metabolic data indicate that MAG is not effective in the maintenance of electrolyte balance when given alone to adrenalectomized patients. The electrolyte data on both serum (Figs. 1 and 2) and urine (Figs. 3 and 4) also show this to be the case. However, in conjunction with small doses of hydrocortisone, MAG in a daily dosage of 4.0 Gm. is quite potent. Furthermore, MAG had no effect on the eosinophil count or the plasma cholesterol level. The steady decline in the cholesterol values was closely related to the hydrocortisone dosage. The possibility of diet playing a role in determining the plasma cholesterol level must be considered. However, it is apparent in both patients that, though a period of loss of appetite occurred, this was only short lived and on the occasions when the patients were receiving 10 mg. of hydrocortisone in conjunction with 4.0 Gm. of MAG per day, their appetites were as voracious as at any time throughout the entire period of study. It is probable that these observations on cholesterol values are similar to those of Di Luzio *et al.* (8), who showed that plasma lipid levels were increased when adrenalectomized dogs were given large doses of cortisone in addition to desoxycorticosterone acetate.

SUMMARY

Two adrenalectomized patients who were given mono-ammonium glycyrrhizinate (MAG) in an oral dosage of 4 Gm. daily were not maintained in a satisfactory clinical state.

The desoxycorticosterone acetate-like action of MAG, observed in subjects with intact adrenals, was absent.

When as little as 10 mg. of hydrocortisone per day was given orally with 4.0 Gm. of MAG, both adrenalectomized patients were maintained in excellent clinical condition. Reduction of the dosage of MAG from 4.0 Gm. to 2.0 Gm. per day was followed by a deterioration in the clinical condition of the patients as well as an increase in sodium excretion. When the dosage of hydrocortisone was raised to 40 mg. daily, the administration of 2.0 Gm. of MAG daily resulted in marked sodium retention. Thus, hydrocortisone and mono-ammonium glycyrrhizinate have synergistic effects in the adrenalectomized patient.

Eosinophil counts and plasma cholesterol values were unaffected by mono-ammonium glycyrrhizinate. Plasma cholesterol values were positively related to the dosage of hydrocortisone.

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THE EFFECT OF LEONTICE EVERSMANNII SAPONINS AND GLYCYRRHIZIC ACID ON EXPERIMENTAL ATHEROSCLEROSIS IN RABBITS

From the references, it is known that the saponins of various plants decrease the cholesterol level in the blood of rabbits with experimental atherosclerosis (and) relieve the clinical course of the illness in sick persons [7, 8].

According to materials by L. N. Sokolova [6], saponins from the Caucasian yam, in a dosage of 5-10 mg./kg. internally, decrease the cholesterol level in the blood of rabbits with alimentary cholesteric atherosclerosis from 455 mg.% to the normal level - 67 mg.%.

A. S. Gladkikh [4] shows that saponins from the Georgian cyclamen also decrease the cholesterol level in the blood of rabbits with experimental atherosclerosis.

Saponins of the Caucasian yam (diosponin) have been found to be effective agents for the treatment of atherosclerosis in the clinical practice [9].

We have studied the effect of leontice saponins and glycyrrhizic acid on cholesterolemia and the development of experimental atherosclerosis in animals.

Leontice eversmannii saponins are produced in the laboratory of glycosides (and) plant matter chemistry at the Uzbek Academy of Sciences by L. G. Mzhel'skaya, V. K. Yatsyn, (and) N. K. Abubakirov [5].

In 1959, N. K. Abubakirov and V. K. Yatsyn extracted glycyrrhizic acid, relating to triterpene saponins, from the roots of Glycyrrhiza glabra L. [1, 2].

We have studied monopotassium salts in glycyrrhizic acid. This light powder is of a yellowish color (and) sweetish in taste.

<u>Procedure</u>. Experimental atherosclerosis was produced in 22 Chinchilla-type rabbits of both sexes, weighing 2.5 -

0.3 kg., using the method of N. N. Anichkov. Cholesterol was injected internally into all the animals (0.3 g./kg. in a 10% solution of sunflower oil) daily over the course of 60 days; furthermore, one group of rabbits was injected internally with leontice saponins (10 mg./kg.), dissolved in distilled water ex tempore, and another (group) - with glycyrrhizic acid (15 mg./kg.), (also) dissolved in distilled water ex tempore. A clinical observation, carried out on the animals during testing, took general condition and weight into account.

Cholesterol content in the blood was determined by (the method of) Engel'gardt and Smirnova [10], lecithin - using Burl's method, on the FEK-M Eikonogen photoelectrocolorimeter.

After completion of the testing, the animals were killed and the degree of development of aortic atherosclerosis was determined [3].

Results of the study. Data from two prophylactic series of tests for studying the effect of leontice saponins and glycyrrhizic acid on the biochemical and morphological indices of experimental atherosclerosis in rabbits are presented in the table.

In rabbits of the second group, the introduction of Leontice eversmannii saponins, in a dosage of 10 mg./kg., decreased cholesterol content in the blood by 33% over 60 days, as compared to the controlled group. In the third group of rabbits, the introduction of glycyrrhizic acid, in a dosage of 15 mg./kg., decreased cholesterol content in the blood by 17% over 2 months, as compared to the controlled (group).

In the first (controlled) group of rabbits, recieving only cholesterol (0.3 g./kg.), it's content in the blood increased from 50-75 to 600-775 mg. %. These rabbits, to the end of the test, became more aggressive; their

fur became sparse (and) ruffled; a region of baldness in the area of the neck and chest was observed in several (animals); the skin in these areas was dry and wrinkled.

The effect of saponins on the level of cholesterol and lecithin in the blood of rabbits with cholesteric atherosclerosis

Animal's Number	Before Testing: Cholesterol mg. %	Before Testing: Lecithin mg. %	Before Testing: Chol./Lec. Coefficient	After 60 Days: Cholesterol mg. %	After 60 Days: Lecithin mg. %	After 60 Days: Chol./Lec. Coefficient	Degree of Aortic Affection
1st group of rabbits - controlled (0.3 g./kg. of cholesterol)							
1	50	75	0.6	600	200	3.0	+++
2	50	75	0.6	625	200	3.1	+++
3	50	100	0.5	725	200	3.6	+++
4	62.5	75	0.8	875	230	3.9	++++
5	62.5	125	0.5	725	207.5	3.4	++
6	60	75	0.8	875	245	3.6	++++
7	87	150	0.5	625	187.5	3.3	+++
8	75	75	1.0	775	200	3.8	+++
Average	62± 10.5	93.7± 24.5	0.66± 0.14	728± 90.3	280.5± 66.7	3.5± 0.36	
2nd group (0.3 g./kg. cholesterol + 10 mg./kg. leontice saponins)							
1	75	125	0.6	400	245	1.5	-
2	60	100	0.6	500	260	1.9	-
3	50	75	0.66	525	245	2.1	+
4	57	125	0.45	525	230	2.2	-
5	50	75	0.66	500	200	2.5	+
6	75	125	0.6	500	260	1.9	++
Average	61.1± 11.8	104.1± 25.7	0.59± 0.05	491.6± 48.3	233± 30.8	2.1± 0.35	
3rd group (0.3 g./kg. cholesterol + 15 mg./kg. glycyrrhizic acid)							
1	60	125	0.4	800	250	3.2	++
2	75	75	1.0	650	230	2.8	+++
3	75	125	0.6	700	200	3.5	+
4	82	150	0.5	600	150	4.0	+++
5	75	100	0.75	525	200	2.6	++
6	50	75	0.66	500	225	2.2	+++
7	50	100	0.5	500	175	2.9	+
8	60	125	0.4	600	175	3.4	++
Average	65.8± 11.9	103.1± 21.7	0.54± 0.16	609.3± 84.5	200.6± 27.9	3.0± 0.47	

Annotation. Small lipoid deposits in the form of minute isolated odonts +, presence of several larger odonts ++, a greater amount of larger odonts +++, diffuse deposits of lipoids in the aortic wall ++++.

In rabbits of the second group, receiving cholesterol (0.3 g./kg.) and leontice saponins (10 mg./kg.), cholesterol content in the blood amounted to 400-500 mg. % till the termination of testing.

In rabbits of the third group, receiving cholesterol (0.3 g./kg.) and glycyrrhizic acid (15 mg./kg.), cholesterol content in the blood was 500-600 mg. % till the end of testing.

Rabbits of the second and third groups withstood the prolonged introduction of saponins well; they gained weight; their fur was thick (and) smooth.

During a macroscopic investigation of rabbits receiving only cholesterol, atherosclerotic changes were expressed in the form of diffuse deposition of lipoids in the aortic intima, particularly in the (aortic) arch and pectoral region, and in the form of large multiple odonts. Atherosclerotic changes were also noted in the semilunar valves.

In rabbits receiving cholesterol and leontice saponins, changes in the aorta were less marked. Minute isolated atherosclerotic odonts in a small quantity (were noted) and, in some rabbits, atherosclerotic changes of any kind in the aorta were entirely undetected.

Atherosclerotic affections of the aorta in rabbits of the third group, which received cholesterol and glycyrrhizic acid in a dosage of 15 mg./kg., were markedly stronger than in rabbits of the second group. In some rabbits of the third group, the aortae were affected with large odonts, distributed along the entire aorta.

Thus, leontice saponins and glycyrrhizic acid show a positive effect on the course of experimental atherosclerosis in rabbits. They decrease the amount of cholesterol in the blood (and) reduce the cholestero-lecithinic coefficient (to the greatest extent, under the effect of leontice saponins). They

facilitate a clinical picture of the course of experimental atherosclerosis (and) decrease the deposition of lipoids in the aorta. The best effect during this experiment is observed from leontice saponins.

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THE PHARMACOLOGICAL PROPERTIES OF GLYCYRRHETINIC ACID—A NEW ANTI-INFLAMMATORY DRUG

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A study of the pharmacological properties of glycyrrhetinic acid, or glycyrrhetic acid, a new anti-inflammatory drug from liquorice, shows it has an extremely low toxicity and is non-irritant to the skin. It has no adverse effects on the heart, circulation or respiration and shows no glucocorticoid-like activity. In large doses in animals it produces water retention, slight sodium retention and an increased excretion of potassium. These effects are not seen with smaller doses used in man.

Glycyrrhetinic or glycyrrhetic acid, a triterpenoid obtained from liquorice, has been proved to be an anti-inflammatory agent[1]. The toxicological properties and pharmacodynamics of this drug are now described. Previously there have been no detailed reports on the pharmacology of this compound, most of the published literature referring to liquorice extract and glycyrrhizin. Molhuysen and others[2] found that a liquorice extract had a deoxycortone-like action, promoting the retention of sodium and water and increasing the excretion of potassium in normal persons. Groen and others[3] reported that liquorice and glycyrrhizinic acid maintained two patients with Addison's disease in correct electrolyte balance and a similar result was obtained by Calvert[4]. Pelser and others[5] found that glycyrrhetinic acid was more effective than glycyrrhizinic acid in this condition.

Liquorice extract was ineffective in one severe case of Addison's disease which had previously shown no response to ACTH[2]. While glycyrrhetinic acid potentiated the action of cortisone[6], alone, it was unable to maintain adrenalectomised patients. Glycyrrhizin also was unable to effect adequate maintenance of the patient with bilateral adrenalectomy[7].

Although glycyrrhetinic acid was thought to have deoxycortone-like actions, Galal[8] has shown that its antidiuretic action differs from that of deoxycortone in rats. Glycyrrhetinic acid has no glucocorticoid-like activity and Hems[9] showed it to be inactive in the mouse liver glycogen test. Recently Atherden[10] found glycyrrhetinic acid to inhibit the metabolism of progesterone and 11-deoxycorticosterone by rat-liver homogenates.

Materials. The glycyrrhetinic acid (Fraction "S") used in these investigations was supplied by Biorex Laboratories Ltd. It was used in the form of tablets and as a saline suspension.

Methods

Acute Toxicity

The acute toxicity of glycyrrhetinic acid was determined on albino mice of both sexes. Injections were made on a weight basis into animals

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weighing between 18 and 22 g. which had fasted overnight. For all routes of administration, where possible, the regression of mortality per cent as probits on the logarithm of the dose was found and the LD50 and limits of error (P 0·95) calculated by the method of Finney[11].

Subacute Toxicity

The subacute toxicity of glycyrrhetinic acid was determined on young rats, which were injected intramuscularly three times a week for 4 weeks. Their weights were determined twice weekly and finally the rats were killed and examined pathologically. Histological sections of the major organs were prepared. The adrenal glands were weighed and frozen sections stained for lipid with Sudan III.

Dermal Toxicity

This was determined in rabbits as described under the "Procedures for the Appraisal of the Toxicity of Chemicals in Foods, Drugs and Cosmetics"[12]. The primary irritation of the skin was measured by an examination of the skin of an albino rabbit after treatment with glycyrrhetinic acid as follows. Small pellets of cotton-wool were saturated with 0·5 ml. of a suspension of glycyrrhetinic acid containing 100 mg. ml. Three of these saturated pellets were then fixed with adhesive plaster to the previously shaven skin of a rabbit's back. The trunk of the animal was then wrapped in a plastic film to minimise evaporation. The skin underneath the pellets was examined after 24 and 72 hours. In another rabbit the skin was abraded before the pellets were applied.

Pharmacodynamics

The pharmacological effects of glycyrrhetinic acid on the cardiovascular system, and on the central and autonomic nervous systems, were studied in mice, rats and anaesthetised cats. The cats were anaesthetised with chloralose (80 mg. kg.), the blood pressure was recorded from the carotid artery and the respiration was recorded from a tracheal cannula by the method described by Paton[13]. Glycyrrhetinic acid, having a low water solubility, could not be injected intravenously, therefore it was injected intraperitoneally or directly into the duodenum. The effects on gastrointestinal motility were studied *in vitro* on the isolated duodenum of the rabbit, and *in vivo* by the transport of a charcoal meal in mice as described by Bryant and others[11].

Urinary System

The effects of glycyrrhetinic acid on the secretion of urine and the excretion of sodium and potassium were studied in rats and anaesthetised cats. An experiment was also made in student volunteers. After an injection of glycyrrhetinic acid, rats were given 10 ml. of water orally per 100 g. body weight and randomly distributed into groups of three or six and placed into metabolism cages. Control groups were given saline. The urine was collected and measured hourly over 5 hours and the sodium and potassium estimated by flame photometry. Cats were anaesthetised

with chloralose and the bladder cannulated through the urethra for collection of the urine. After a 75 minute control period 100 mg./kg. of glycyrrhetinic acid was injected intraperitoneally. The urine was collected over another 60 minutes. The volumes of urine excreted were measured at 15 minute intervals and taken for estimation of the sodium and potassium present. Samples of blood were also taken at these times for estimation of sodium, potassium, total chloride and glycyrrhetinic acid in the serum.

Glycyrrhetinic acid was estimated in the serum by a modification of the method described by Van Katwijk and Huis in't Veld[15] for the determination of glycyrrhetinic acid in urine. Serum or plasma (0·2 ml.) was added to 0·1N sulphuric acid (1 ml.) and the mixture extracted three times with ether (3 × 2 ml.). The combined ethereal solution was then extracted with 0·5N ammonium hydroxide solution (2 ml.) and the ether layer discarded. The alkaline layer was acidified with 0·7 ml. of 2N sulphuric acid and extracted three times with ether. The combined ether extract was evaporated in a current of air and the residue dried over silica gel at 20° and 3 mm. The residue was dissolved in 3 ml. 95 per cent spectroscopically pure ethanol and the optical density measured at 248 mμ in a spectrophotometer. Serum from the same animal collected immediately before the administration of glycyrrhetinic acid was similarly extracted and the ethanolic solution of the final residue was used as a reference blank. A calibration curve was prepared by measuring the optical density of solutions of glycyrrhetinic acid in 95 per cent ethanol containing 1 to 40 μg. 3 ml. The curve was linear throughout this range. The accuracy of the method was checked by adding known concentrations of glycyrrhetinic acid to a control sample of the serum. In a typical experiment in which 5·0 μg. and 10·0 μg. were added to 0·2 ml. serum samples, recoveries of 4·4 μg. (88 per cent) and 10·1 μg. (101 per cent) respectively were obtained. To ensure that the optical density at 248 mμ was specific for glycyrrhetinic acid, measurements were made over the wavelengths 230 to 270 mμ. Maximum absorption at 248 mμ was found unless the serum specimen was haemolysed, in which case non-specific absorption was observed.

The effects of an oral dose of glycyrrhetinic acid was determined in eight healthy male student volunteers in a blind cross over trial. Each student was given 0·2 g. or 0·5 g. of glycyrrhetinic acid or a dummy tablet and 30 minutes later drank 1500 ml. of water. The urine was collected at 30-minute intervals over 2½ hours and the volume was recorded.

Glucocorticoid Action

This was tested in adrenalectomised mice submitted to a cold stress[16]. Groups of 10 mice were adrenalectomised under ether anaesthesia. The following day one group was injected intraperitoneally with 170 mg. kg. of glycyrrhetinic acid and the other group with saline as the controls. Their survival times in a refrigerator at 4° were then recorded to the nearest half hour.

Results

Acute Toxicity

Glycyrrhetinic acid had a low toxicity. Given orally to mice no deaths occurred following single doses as high as 610 mg./kg., which was the maximal dose that could be administered. Similarly by the subcutaneous route it was not possible to kill any mice at this dose level. By the intraperitoneal route deaths did occur over a period of 48 hours and the LD50 was 308 mg. kg. with fiducial limits of error (P = 0·95) from 279 to 340 mg. kg. (Table I). High doses caused sedation, palor of the extremities, and respiratory depression. Death was usually delayed, the mice generally dying on the second day after the administration. Pathologically there was evidence of peritonitis, probably caused by the presence of the insoluble glycyrrhetinic acid in the peritoneal cavity. Glycyrrhetinic acid could not be given intravenously because of its low solubility in water.

TABLE I

ACUTE INTRAPERITONEAL TOXICITY OF GLYCYRRHETINIC ACID IN ALBINO MICE

Dose mg. kg.	No. of mice	Deaths (after 2 days)
216	20	3
263	20	6
320	20	10
390	20	16

LD50 = 308 mg. kg. Fiducial limits (P = 0·95) 279 to 340 mg./kg.

Subacute Toxicity

The growth of young rats was not depressed by intramuscular injections of 10 and 20 mg. of glycyrrhetinic acid three times a week. The treated rats maintained good health, ate well and grew as well as the untreated controls. When killed after 4 weeks there was no evidence at post-mortem of any gross pathological effects and histological sections of the major organs showed no abnormalities. There was no adrenal atrophy, as occurs with cortisone, and sections of the glands were normal except for a slight thinning of the lipid in the zona glomerulosa. This was by no means as severe as occurs with deoxycortone.

TABLE II

THE EFFECT OF GLYCYRRHETINIC ACID ON THE EXCRETION OF WATER IN RATS

Treatment	Volume of urine excreted in ml. Hours after water administration				
	1	2	3	4	5
1. Saline controls	3·0*	7·0	8·3	8·7	9·3
2. Glycyrrhetinic acid 125 mg./kg. Water 30 min. after injection	0·8	4·2	7·2	7·3	8·0
3. Glycyrrhetinic acid 125 mg./kg. Water 2 hours after injection	3·7	7·8	8·3	8·6	9·5
4. Glycyrrhetinic acid 125 mg./kg. Water 4 hours after injection	2·5	9·2	9·5	9·8	10·0

* Each figure represents the mean volume from three rats.

Dermal Toxicity

There was no evidence of oedema or erythema of the normal or abraded skin, proving that glycyrrhetinic acid has no primary irritant action on the skin of the rabbit.

Pharmacodynamics

Glycyrrhetinic acid had no untoward effects on the central or autonomic nervous systems, nor on the heart and circulation.

The central nervous system. This was affected only by extremely large doses of glycyrrhetinic acid. In the mouse a dose of 25 mg. (1250 mg. kg.)

TABLE III

THE EFFECT OF GLYCYRRHETINIC ACID ON THE URINARY EXCRETION OF SODIUM AND POTASSIUM IN RATS

Treatment		Sodium excretion		Potassium excretion	
		mg. 5 hr.	Per cent controls	mg. 5 hr.	Per cent controls
1. Saline controls		3·2*		3·8	
2. Glycyrrhetinic acid 125 mg. kg.	Water 30 min. after injection	0·9	29	4·8	125
3. Glycyrrhetinic acid 125 mg. kg.	Water 2 hours after injection	3·8	117	7·7	200
4. Glycyrrhetinic acid 125 mg. kg.	Water 4 hours after injection	2·8	86	6·0	157

* Each figure represents the mean from three rats.

intraperitoneally caused sedation, hypnosis, hypothermia and respiratory depression.

The autonomic nervous system. In the mouse intraperitoneal and oral doses of 25 mg. or 12 mg. subcutaneously did not stimulate or depress

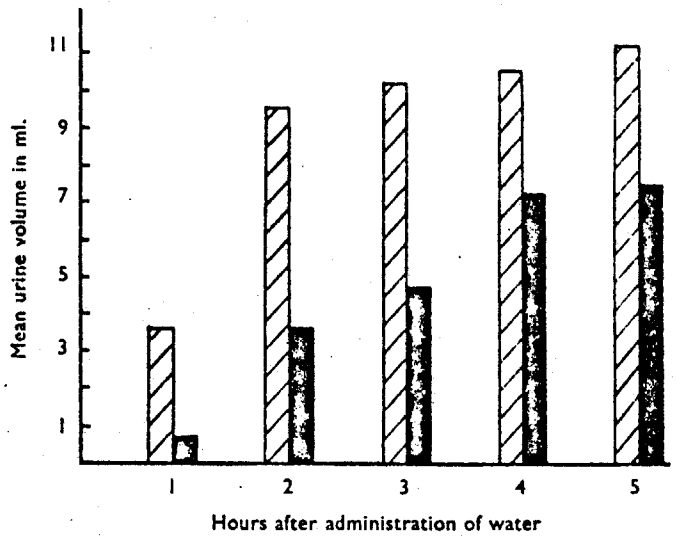

FIG. 1. The antidiuretic action of glycyrrhetinic acid (125 mg. kg. i.p.) in rats given 100 ml. kg. of water orally. Each value is the mean from six rats. Shaded area, controls; black area glycyrrhetinic acid.

either the sympathetic or parasympathetic branches of the autonomic nervous system. Similarly in the cat, an intraperitoneal dose of 125 mg./kg. did not alter the blood pressure or affect the normal responses to stimulation of the sympathetic or parasympathetic nerves. The responses

to an intravenous injection of acetylcholine, nicotine or adrenaline were normal.

The cardiovascular system. In the anaesthetised cat very large doses (125 mg. kg.) administered intraperitoneally or injected directly into the duodenum did not affect the blood pressure or the heart beat. Intravenous administration of glycyrrhetinic acid was precluded because of its low solubility.

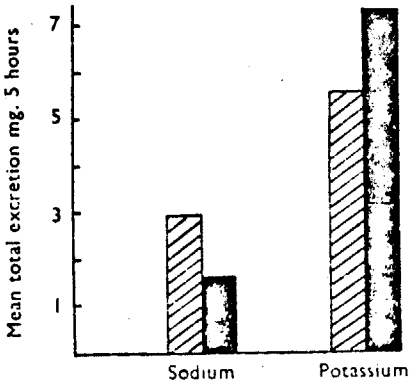

FIG. 2. The effect of glycyrrhetinic acid (125 mg. kg. i.p.) on the urinary excretion of sodium and potassium in rats. Each value is the mean from six rats. Shaded area, controls; black area, glycyrrhetinic acid.

The respiratory system. In the anaesthetised cat doses as high as 125 mg. kg., injected intraperitoneally, did not affect the depth or rate of respiration. In mice respiratory depression was only seen after toxic doses of glycyrrhetinic acid (610 mg./kg.) were given intraperitoneally.

The gastrointestinal tract. Glycyrrhetinic acid did not affect the motility of the gastrointestinal tract. *In vitro* the addition of 1 mg. of glycyrrhetinic acid to a 15 ml. bath did not affect the tone or contractions of the isolated duodenum of the rabbit. The normal responses to acetylcholine, adrenaline and barium chloride were unchanged, showing the absence of a spasmolytic action. *In vivo* the rate of transport of carbon through the stomach and intestine of the mouse was not affected when compared with untreated controls. The oral administration of glycyrrhetinic acid in rats and mice did not have a constipating action, or cause diarrhoea.

TABLE IV

THE EFFECTS OF GLYCYRRHETINIC ACID ON THE EXCRETION OF URINE, SODIUM AND POTASSIUM IN THE CAT

Urine sample (15 min. intervals)	Volume ml.	Sodium		Potassium	
		m. eq./l.*	m. eq./15 min.	m. eq./l.	m. eq./15 min.
1	4·8	210	—	30	—
2	3·2	205	0·66	26·5	0·085
3	2·6	205	0·53	26	0·068
4	2·3	195	0·45	25·5	0·059
5	3·4	185	0·63	27·5	0·063
	Injection of 200 mg./kg. of glycyrrhetinic acid				
6	3·5	170	0·60	31·5	0·110
7	2·0	180	0·36	32	0·064
8	1·7	175	0·30	28	0·048
9	1·2	185	0·22	28	0·038.

* Milliequivalents per litre urine.

The urinary system. Glycyrrhetinic acid did have an effect on kidney function in the rat. This has been examined in some detail. There was a marked antidiuretic action, which confirms the work of Galal[8], a retention of sodium and an increase in the urinary potassium excretion.

Typical results are shown in Tables II and III. In this experiment group 1, the controls, were given normal saline, while groups 2, 3 and 4 were injected intraperitoneally with 125 mg. kg. of glycyrrhetinic acid. The corresponding groups of rats were given an oral dose of 10 ml. 100 g. of water either half, 2 or 4 hours after the administration of glycyrrhetinic acid. The effects on sodium and water metabolism occurred in group 2.

TABLE V

THE EFFECTS OF GLYCYRRHETINIC ACID ON SODIUM, POTASSIUM AND CHLORIDES IN THE SERUM OF THE CAT

Treatment	Glycyrrhetinic acid mg./kg.	Mean serum Sodium	Potassium	Chlorides
A. Control (30 min. before glycyrrhetinic acid)		150	3·4	124
B. Control (15 min. before glycyrrhetinic acid)		148	3·4	124
C. 15 min. after glycyrrhetinic acid	0·25	154	3·6	127
D. 75 min. after glycyrrhetinic acid	0·25	153	3·8	125

TABLE VI

EFFECTS OF GLYCYRRHETINIC ACID ON URINE SECRETION IN EIGHT MALE VOLUNTEERS AFTER DRINKING 1500 ML. OF WATER

Treatment	Time in hours after drink of water				
	0		1½	2	2½
Placebo controls	69*	264	808	792	958
Glycyrrhetinic acid 0·2 g.	65	250	658	728	956
Glycyrrhetinic acid 0·5 g.	124	426	731	631	1,086

* Average cumulative total excretion in ml.

TABLE VII

GLUCOCORTICOID ACTIVITY OF GLYCYRRHETINIC ACID IN THE MOUSE SURVIVAL TEST

Treatment (dose 20 g. mouse)	Mean survival time in hours	Standard error
Controls saline	4·90*	0·45
Glycyrrhetinic acid 1·56 mg.	3·55	0·40
" " 6·25 mg.	2·05	0·20
" " 25 mg.	2·65	0·36

* Each value is the mean of ten mice.

where the administration of glycyrrhetinic acid preceded the water loading by 30 minutes, and not in groups 3 and 4 where the time interval was longer. Potassium excretion was increased in all groups.

These results were confirmed in a second experiment using two groups of six rats. The first group received 0·25 ml. of saline and the second group 125 mg. kg. of glycyrrhetinic acid intraperitoneally. 10 ml. of water per 100 g. rat was given orally 30 minutes later. The urine excretion is shown in Figure 1 and the excretion of sodium and potassium in Figure 2.

In the anaesthetised cat an intraperitoneal injection of 100 mg. kg. of glycyrrhetinic acid caused a reduction in the urine flow, a slight decrease in the urinary excretion of sodium and a slight increase in the excretion of potassium (Table IV). There were no significant changes in the serum concentrations of sodium, potassium and total chlorides (Table V).

Estimation of glycyrrhetinic acid in the serum showed that absorption occurred from the peritoneal cavity.

In eight student volunteers oral doses of 0·2 g. and 0·5 g. of glycyrrhetinic acid before drinking 1500 ml. of water did not produce an antidiuretic effect (Table VI).

Glucocorticoid action. Glycyrrhetinic acid did not increase the survival time of adrenalectomised mice submitted to a cold stress (Table VII). This confirms the observations of Wendel and others[11] and D'Arcy and others[12]. Hems[9] was unable to find a glucocorticoid action with glycyrrhetinic acid when tested by the liver glycogen test.

CONCLUSIONS

Glycyrrhetinic acid is seen to have a remarkably low toxicity and therefore can be applied to the skin with complete safety in dermatological conditions. So far it has been little used internally, although liquorice extract has been taken orally for years. We have confirmed that exceptionally large doses of glycyrrhetinic acid in animals have an antidiuretic action associated with changes in the metabolism of sodium and potassium; but do not cause kidney damage. Water retention was not seen with small doses used in human volunteers. The low solubility of glycyrrhetinic acid in body fluids has so far precluded parenteral administration in man, but this will be possible with development of more soluble derivatives which may prove of value in rheumatic diseases. An important property of glycyrrhetinic acid is its complete freedom from glucocorticoid-like actions, a serious disadvantage with the corticosteroids. While it has been shown that little absorption of these steroids occurs through normal skin this cannot be assumed in dermatological conditions where the protective dermal layers may be broken. Much remains to be discovered about the mode of action of glycyrrhetinic acid, but it offers a new approach to the treatment of inflammatory conditions free from the disadvantages of corticoids which have claimed so much attention and disproves the concept that an anti-inflammatory agent must of necessity have a concomitant corticoid-like action.

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FINAL REPORT

Submitted to: DHEW/Public Health Service
Food and Drug Administration CA-272
5600 Fishers Lane-Room 5C-13
Rockville, Maryland 20852

Date: February 15, 1972

Laboratory No. 0728 b
Contract No. FDA 71-260

Sample: Fine dark brown powdered material.

Marking: FDA 71-1 (Ammonium glycyrrhizinate)

Examination Requested: Teratologic evaluation of FDA 71-1 in mice.

Procedure: See Appendix I

Results: See Tables 1 through 4 and Appendix II

Conclusion:

Attention is called to the fact that this is the first of a series of reports which will be issued in accordance with the terms of the contract cited above. Eventually, a total of at least 36 compounds will have been tested in 18 pairs; each pair being run concurrently against one sham-treated control and one positive control group. Because of the inherent variability of biological data of the type dealt with here, the accumulation and pooling of sequential sets of control values will greatly enhance the statistical value of the findings and the ultimate reliability of the test results.

For these reasons, the conclusion stated below is regarded as provisional and subject to reexamination in the light of later findings:

"The administration of up to 1000 mg/kg (body weight) of the test material to pregnant mice for 10 consecutive days had no clearly discernible effect on nidation or no maternal or fetal survival. The number of abnormalities seen in either soft or skeletal tissues of the test groups did not differ from the number occurring spontaneously in the sham-treated controls."

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Kenneth Morgareidge

Kenneth Morgareidge, Ph.D.
Vice President

This report is submitted for the exclusive use of the person, partnership, or corporation to whom it is addressed, and neither the report nor the name of these Laboratories nor of any members of its staff, may be used in connection with the advertising or sale of any product or process without written authorization.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups: 1 through 6

Material: FDA 71-1

Date February 15, 1972

Laboratory No. 0728 b

Table 1

Fate Summary
(Mice)

Group	Material	Dose mg/kg	Total		At Term	
			Mated	Pregnant	Surviving (Total)	Number Pregnant
1	Sham	0	27	20	25	19
2	Aspirin*	200	30	26	20	14
3	FDA 71-1	27	28	20	24	19
4	FDA 71-1	90	30	24	27	23
5	FDA 71-1	300	30	24	25	22
6	FDA 71-1	1000	25	21	24	21

* Positive Control

Group: 1 through 6

Material: FDA 71-1

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Table 2

Reproduction Data
(Mice)

Date February 15, 1972

Laboratory No. 0728 b

Group:	1	2	3	4	5	6
Dose (mg/kg):	Sham	Aspirin*	27	90	300	1000
Number of females:						
Total pregnant	20	26	20	24	24	21
Pregnant at term	19	14	19	23	22	21
Number of live litters:	20	14	19	23	22	21
Number of implant sites:						
Total	247	308	251	289	283	273
Average/pregnant dam	12.4	11.8	12.6	12.0	11.8	13.0
Number of fetuses alive:						
Total	240	165	221	252	252	260
Average/live litter	12.0	11.8	11.6	11.0	11.5	12.4
Average/pregnancy at term	12.6	11.8	11.6	11.0	11.5	12.4
Number of fetuses dead:						
Total	5	49	3	10	9	2
Litters with one or more dead	4	6	2	9	3	2
Pregnancies at term (%)	21.1	42.9	10.5	39.1	13.6	9.5
Litters with all dead	0	4	0	0	1	0
Pregnancies at term (%)	0	28.6	0	0	4.5	0
Number of resorptions:						
Total:	2	94	27	27	9	11
Litters with one or more resorptions	1	12	9	8	8	8
Pregnancies at term (%)	5.3	85.7	47.4	34.8	36.4	38.1
Litters with total resorptions	0	8	1	1	0	0
Pregnancies at term (%)	0	57.1	5.3	4.3	0	0
Average fetus weight, g.	0.97	0.87	0.93	0.93	0.99	0.98

* Positive control at 200 mg/kg

Groups 1) ugh 6

Material FDA 71-1

T.)e 3

Summary of Skeletal Findings
(Mice)

Laborator) . 0728b

Date February 15, 197:

Findings	Group No: 1	2 **	3	4	5	6
	Dose (mg/kg): Sham	Aspirin	27	90	300	1000
Live Fetuses Examined	163/19	115/14	155/19	186/23	179/22	181/21
Fetuses with:						
Incomplete sternebrae	14/6	20/7	58/18	81/22	24/12	45/14
Scrambled sternebrae				1/1		10/1
Bipartite sternebrae	123/17	75/14	110/19	151/23	91/19	106/18
Missing sternebrae						
Fused sternebrae						
Extra sternebrae	3/3	1/1				
Incomplete hyoid						5/3
Fetuses with:						
Scrambled vertebrae						
Tail defects (short, scrambled, etc.)	1/1					
Incomplete ossification; vertebrae	137/17	57/10	87/13	99/18	66/15	81/18
Fetuses with:						
Fused ribs				3/1		
Incomplete ribs		6/1	1/1			
Wavy ribs	1/1					
Less than 12 ribs						
More than 13 ribs	2/2	5/2	5/3	6/3		5/3
Other Findings:						
Scoliosis						
Delayed cranial ossification	25/7	32/9	11/5	17/5	16/5	41/9
Craniostosis						
Feet; retarded ossification	115/16	35/4	97/11	87/15	73/11	156/19
Meningocele			1/1	1/1		

*Numerator = Number of fetuses affected; Denominator = number of litters affected

** Positive control at 200 mg/kg

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 1 through 6

Species Mice

Date February 15, 1972

Laboratory No. 0728 b

Table 4

Average Body Weights *

Group	Material	Dose Level	Day 0	6	11	15	17**
		mg/kg	g				
1	Sham	0	29.3	31.4	34.6	44.1	49.6 (19)
2	Aspirin	200	28.8	31.0	31.1	36.1	44.8 (14)
3	FDA 71-1	27	29.0	31.4	34.7	43.6	48.7 (19)
4	FDA 71-1	90	29.7	31.8	35.6	41.9	49.8 (23)
5	FDA 71-1	300	29.6	32.5	35.9	45.2	50.9 (22)
6	FDA 71-1	1000	30.4	32.7	36.5	45.9	52.2 (21)

* Of pregnant dams
** Number of surviving dams in parentheses (c.f. Table 1).

Appendix I

Teratology Study in Mice

Virgin adult female albino CD-1 outbred mice were individually housed in disposable plastic cages in temperature and humidity-controlled quarters with free access to food and fresh tap water. They were mated with young adult males, and observation of the vaginal sperm plug was considered Day 0 of gestation. Beginning on Day 6 and continuing daily through Day 15 of gestation, the females were dosed with the indicated dosages by oral intubation; the controls were sham treated.

Body weights were recorded on Days 0, 6, 11, 15, and 17 of gestation. All animals were observed daily for appearance and behavior with particular attention to food consumption and weight, in order to rule out any abnormalities which may have occurred as a result of anorexic effects in the pregnant female animal.

On Day 17 all dams were subjected to Caesarean section under surgical anesthesia, and the numbers of implantation sites, resorption sites, and live and dead fetuses were recorded. The body weights of the live pups were also recorded. The urogenital tract of each dam was examined in detail for anatomical normality.

All fetuses were examined grossly for the presence of external congenital abnormalities. One-third of the fetuses of each litter underwent detailed visceral examinations employing 10X magnification. The remaining two-thirds were cleared in potassium hydroxide (KOH), stained with alizarin red S dye and examined for skeletal defects.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 1

Material Sham

Dose 0

Appendix II

Reproduction Data in Mice (Individual)

Date February 15, 1972

Laboratory No. 0728

Dam No.	Fate *	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
S 8001	P	18	18	0	0	0.91	
S 8002	P	12	12	0	0	0.89	
S 8003	P	15	15	0	0	0.63	
S 8004	NP	0				--	Died Day 16
S 8005	P	14	12	2	0	1.05	
S 8006	P	14	14	0	0	0.96	
S 8007	P	14	14	0	0	1.03	
S 8008	P	9	9	0	0	0.79	
S 8009	P	11	11	0	0	1.31	
S 8010	P	12	11	1	0	0.95	
S 8011	P	13	13	0	0	0.89	
S 8012	NP	0				--	
S 8013	P	10	10	0	0	0.87	
S 8014	NP	0				--	
S 8015	P	13	13	0	0	0.85	
S 8016	P	15	15	0	0	0.75	
S 8017	P	11	11	0	0	1.00	
S 8018	P	12	11	1	0	0.81	
S 8019	P	9	9			--	Dam died day 14
S 8020	P	16	14	0	2	0.98	
S 8021	NP	0				--	
S 8022	NP	0				--	
S 8023	NP	0				--	
S 8024	NP	0				--	
S 8025	P	6	6	0	0	1.53	
S 8026	P	13	13	0	0	1.15	
S 8027	P	10	9	1	0	1.12	

* P= Pregnant; NP= Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 2

Material Aspirin

Dose 200 mg/kg

Appendix II

Reproduction Data in Mice (Individual)

Date February 15, 1972

Laboratory No. 0728

Dam No.	Fate *	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
A 8001	P	12	0	12	0	--	Died day 15.
A 8002	P	10	10	0	0	0.74	
A 8003	NP	0				--	
A 8004	P	12	0	0	12	--	Died day 16.
A 8005	P	7	0	0	7	--	
A 8006	P	2	0	0	2	--	
A 8007	P	14	13	0	1	0.80	
A 8008	P	13	0	0	13	--	Died day 12.
A 8009	P	11	0	0	11	--	Died day 17.
A 8010	P	16	0	0	16	--	Died day 12.
A 8011	P	13	10	0	3	0.69	
A 8012	P	13	0	0	13	--	Died day 11.
A 8013	P	14	0	0	14	--	Died day 14.
A 8014	P	13	13	0	0	0.74	
A 8015	P	12	0	12	0	--	Died day 9.
A 8016	P	12	11	0	1	0.92	
A 8017	P	15	0	15	0	--	Died day 10.
A 8018	P	12	12	0	0	1.11	
A 8019	P	8	0	8	0	--	Died day 16
A 8020	NP	0				--	
A 8021	P	12	11	1	0	0.81	
A 8022	NP	0				--	
A 8023	P	14	14	0	0	0.83	
A 8024	P	11	11	0	0	1.08	
A 8025	P	8	8	0	0	0.82	
A 8026	P	14	14	0	0	0.88	
A 8027	NP	0				--	
A 8028	P	15	14	1	0	0.89	
A 8029	P	12	12	0	0	1.03	
A 8030	P	13	12	0	1	0.87	

* P= Pregnant; NP= Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 3

Material FDA 71-1

Dose 27 mg/kg

Appendix II

Reproduction Data in Mice (Individual)

Date February 15, 1972

Laboratory No. 0728 b

Dam No.	Fate *	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
B 8001	P	13	13	0	0	1.00	
B 8002	P	11	11	0	0	0.93	
B 8003	P	15	12	2	1	1.06	
B 8004	P	9	9	0	0	0.86	
B 8005	P	11	11	0	0	--	
B 8006	P	16	15	1	0	--	
B 8007	P	13	13	0	0	0.99	
B 8008	NP	0				--	
B 8009	P	15	13	0	2	1.02	
B 8010	NP	0	0	0	0	--	Died day 13.
B 8011	P	12	11	0	1	0.97	
B 8012	P	15	15	0	0	1.15	
B 8013	P	9	9	0	0	0.94	
B 8014	P	14	14	0	0	0.72	
B 8015	NP	0				--	
B 8016	P	12	10	0	2	0.76	
B 8017	P	12	10	0	2	0.89	
B 8018	P	10	9	0	1	0.92	
B 8019	NP	0				--	
B 8020	P	11	10	0	1	0.89	
B 8021	NP	0				--	Died day 12.
B 8022	NP	0				--	Died day 10.
B 8023	NP	0				--	
B 8024	NP	0				--	
B 8025	P	12	12	0	0	0.98	
B 8026	P	12	12	0	0	1.01	
B 8027	P	14	12	0	2	0.78	
B 8028	P	15	0	0	15	--	Died day 10.

* P= Pregnant; NP= Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Appendix II

Reproduction Data in Mice (Individual)

Group 4

Material FDA 71-1

Dose 90 mg/kg

Date February 15, 1972

Laboratory No. 0728 b

Dam No.	Fate *	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
B 8031	P	15	13	0	2	0.97	
B 8032	P	13	12	1	0	0.88	
B 8033	P	11	11	0	0	0.91	
B 8034	NP	0				--	
B 8035	P	15	7	0	8	1.06	
B 8036	P	16	16	0	0	0.95	
B 8037	P	12	12	0	0	1.01	
B 8038	NP	0				--	
B 8039	NP	0				--	Died day 12.
B 8040	NP	0				--	Died day 14.
B 8041	P	9	8	1	0	0.79	
B 8042	P	10	10	0	0	0.89	
B 8043	P	15	14	1	0	0.74	
B 8044	P	10	10	0	0	1.15	
B 8045	P	14	13	0	1	0.82	
B 8046	P	14	13	0	1	1.00	
B 8047	P	11	3	2	6	0.74	
B 8048	P	13	12	1	0	0.75	
B 8049	P	8	7	1	0	0.87	
B 8050	P	13	12	1	0	1.04	
B 8051	P	11	11	0	0	1.07	
B 8052	NP	0				--	
B 8053	NP	0				--	
B 8054	P	12	11	1	0	1.10	
B 8055	P	14	11	0	3	0.93	
B 8056	P	10	10	0	0	0.89	
B 8057	P	14	13	0	1	0.87	
B 8058	P	5	0	0	5	--	Died day 17.
B 8059	P	13	12	1	0	0.96	
B 8060	P	11	11	0	0	1.08	

* P= Pregnant; NP= Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Appendix II

Reproduction Data in Mice (Individual)

Group 5

Material FDA 71-1

Dose 300 mg/kg

Date February 15, 1972

Laboratory No. 0728 b

Dam No.	Fate *	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
B 8061	P	11	11	0	0	0.85	
B 8062	P	3	3	0	0	1.17	
B 8063	NP	0				--	Died day 14.
B 8064	NP	0				--	Died day 14.
B 8065	NP	0				--	Died day 7.
B 8066	P	13	11	0	2	0.88	
B 8067	P	14	14	0	0	0.86	
B 8068	NP	0				--	
B 8069	P	11	10	0	1	1.05	
B 8070	P	15	15	0	0	0.96	
B 8071	P	12	11	0	1	0.85	
B 8072	P	14	14	0	0	1.04	
B 8073	P	12	11	0	1	0.86	
B 8074	P	15	15	0	0	1.09	
B 8075	P	11	11	0	0	1.14	
B 8076	P	14	14	0	0	0.91	
B 8077	P	2	2	0	0	0.96	
B 8078	P	15	11	2	2	0.97	
B 8079	P	8	8	0	0	1.05	
B 8080	P	14	13	0	1	0.98	
B 8081	P	6	0	6	0	--	Died day 10.
B 8082	NP	0				--	
B 8083	P	14	14	0	0	1.04	
B 8084	NP	0				--	
B 8085	NP					--	Died day 8.
B 8086	P	10	10	0	0	1.14	
B 8087	P	14	13	0	1	1.07	
B 8088	P	15	15	0	0	0.98	
B 8089	P	15	14	0	1	0.85	
B 8090	P	12	12	0	0	0.99	

* P= Pregnant: NP= Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 6

Material FDA 71-1

Dose 1000 mg/kg

Appendix II

Reproduction Data in Mice (Individual)

Date February 15, 1972

Laboratory No. 0728 b

Dam No.	Fate *	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
B 8091	NP	0				--	
B 8092	P	13	12	1	0	1.01	
B 8093	P	16	15	0	1	0.96	
B 8094	--	--					Not assigned.
B 8095	--	--					Not assigned.
B 8096	P	12	12	0	0	1.22	
B 8097	P	15	13	0	2	0.87	
B 8098	P	14	11	0	3	0.89	
B 8099	P	17	17	0	0	0.81	
B 8100	P	13	12	0	1	1.15	
B 8101	P	14	14	0	0	0.77	
B 8102	NP	0				--	Died day 15.
B 8103	P	13	13	0	0	0.96	
B 8104	P	11	10	0	1	0.94	
B 8105	P	11	10	0	1	0.87	
B 8106	P	12	12	0	0	1.08	
B 8107	NP	0				--	
B 8108	P	11	11	0	0	1.00	
B 8109	P	10	10	0	0	0.98	
B 8110	P	11	11	0	0	0.96	
B 8111	P	13	12	0	1	0.95	
B 8112	P	14	13	0	1	1.05	
B 8113	P	13	12	1	0	0.95	
B 8114	P	14	14	0	0	0.87	
B 8115	P	12	12	0	0	1.28	
B 8116	NP	0				--	
B 8117	P	14	14	0	0	0.95	

* P= Pregnant; NP= Not Pregnant

Food and Drug Research Laboratories
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FINAL REPORT

Submitted to: DHEW/Public Health Service
Food and Drug Administration CA-272
5600 Fishers Lane-Room 5C-13
Rockville, Maryland 20852

Date: February 15, 1972

Laboratory No. 0729b
Contract No. FDA 71-260

Sample: Fine dark brown powdered material.

Marking: FDA 71-1 (Ammonium glycyrrhizinate).

Examination Requested: Teratologic evaluation of FDA 71-1 in rats.

Procedure: See Appendix I.

Results: See Tables 1 through 4 and Appendix II.

Conclusion: Attention is called to the fact that this is the first of a series of reports which will be issued in accordance with the terms of the contract cited above. Eventually, a total of at least 36 compounds will have been tested in 18 pairs; each pair being run concurrently against one sham-treated control and one positive control group. Because of the inherent variability of biological data of the type dealt with here, the accumulation and pooling of sequential sets of control values will greatly enhance the statistical value of the findings and the ultimate reliability of the test results.

For these reasons, the conclusion stated below is regarded as provisional and subject to reexamination in the light of later findings:

"The administration of up to 1000 mg/kg (body weight) of the test material to pregnant rats for 10 consecutive days had no clearly discernible effect on nidation or on maternal or fetal survival. The number of abnormalities seen in either soft or skeletal tissues of the test groups did not differ from the number occurring spontaneously in the sham-treated controls."

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Kenneth Morgareidge

Kenneth Morgareidge, Ph.D.
Vice President

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups: 1 through 6

Material: FDA 71-1

Date February 15, 1972

Laboratory No. 0729 b

Table 1

Fate Summary
(Rats)

Group	Material	Dose mg/kg	Total		At Term	
			Mated	Pregnant	Surviving (Total)	Number Pregnant
1	Sham	0	24	21	25	21
2	Aspirin *	250	26	20	22	19
3	FDA 71-1	27	24	22	24	21
4	FDA 71-1	90	26	20	23	20
5	FDA 71-1	300	25	21	21	18
6	FDA 71-1	1000	25	22	21	20

* Positive Control

Group: 1 through 6

Material: FDA 71-1

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Table 2

Reproduction Data
(Rats)

Date February 15, 1972

Laboratory No. 0729 b

Group:	1	2	3	4	5	6
Dose (mg/kg):	Sham	Aspirin*	27	90	300	1000
Number of females:						
Total pregnant	21	20	22	20	21	22
Pregnant at term	21	19	21	20	18	20
Number of live litters:	21	19	21	20	21	21
Number of implant sites:						
Total	245	230	220	218	254	254
Average/pregnant dam	11.7	11.5	10.0	10.9	12.1	11.5
Number of fetuses alive:						
Total	242	206	202	199	246	236
Average/live litter	11.5	10.8	9.62	9.95	11.7	11.2
Average/pregnancy at term	11.5	10.8	9.62	9.95	13.7	11.8
Number of fetuses dead:						
Total	0	9	0	0	1	11
Litters with one or more dead	0	1	0	0	1	2
Pregnancies at term (%)	0	5.26	0	0	5.56	10.0
Litters with all dead	0	0	0	0	0	1
Pregnancies at term (%)	0	0	0	0	0	5.0
Number of resorptions:						
Total:	3	15	18	19	7	7
Litters with one or more resorptions	3	8	11	8	4	6
Pregnancies at term (%)	14.3	42.1	52.4	14.0	22.2	30.0
Litters with total resorptions	0	0	1	0	0	0
Pregnancies at term (%)	0	0	4.8	0	0	0
Average fetus weight, g.	4.05	3.55	4.02	3.98	3.64	3.73

* Positive control at 250 mg/kg

Groups hrough 6

Material FDA 71-1

le 3

Summary of Skeletal Findings

(Rats)

Laborato Io. 0729 1

Date February 15, 19

Findings	Group No:	1	2	3	4	5	6
	Dose (mg/kg):	Sham	Aspirin**	27	90	300	1000
Live Fetuses Examined		173/21	148/19	152/21	143/20	149/18	160/20
Fetuses with:							
Incomplete sternebrae		101/19	106/19	97/20	90/19	105/17	115/20
Scrambled sternebrae			2/1		2/2	2/2	
Bipartite sternebrae		7/6	5/5	4/4	6/5	3/3	4/4
Missing sternebrae		39/12	58/14	27/8	24/9	48/17	32/12
Fused sternebrae				8/5			
Extra sternebrae							
Fetuses with:							
Scrambled vertebrae							
Tail defects (short, scrambled, etc.)							
Fetuses with:							
Fused ribs							
Incomplete ribs		1/1	1/1	1/1	1/1		1/1
Wavy ribs		8/6	24/12	11/6	4/3	15/8	20/11
Less than 12					2/1		
Other Findings:							
Scoliosis							
Delayed cranial ossification		20/10	29/11	12/8	10/8	15/7	11/7
Craniostosis			1/1			2/2	
Incomplete ossification of phalanges			2/2	1/1		2/2	1/1

*Numerator = Number of fetuses affected; Denominator = number of litters affected

** Positive control at 250 mg/kg

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 1 through 6

Species Rats

Date February 15, 1972

Laboratory No. 0729 b

Table 4*

Average Body Weights

Group	Material	Dose Level	Day 0	6	11	15	20**
		mg/kg	g				
1	Sham	0	240.5	253.0	265.8	281.3	360.2 (21)
2	Aspirin	250	240.1	251.6	258.8	280.1	335.8 (19)
3	FDA 71-1	27	241.1	247.3	257.4	277.0	337.0 (22)
4	FDA 71-1	90	239.3	248.3	261.8	277.7	337.2 (20)
5	FDA 71-1	300	237.9	247.2	253.7	273.0	343.6 (18)
6	FDA 71-1	1000	239.8	250.5	259.1	275.5	344.1 (20)

* Of pregnant dams
** Number of surviving dams in parentheses (c.f. Table 1).

Appendix I

Teratology Study in Rats

Virgin adult female albino rats (Wistar derived stock) were individually housed in mesh bottom cages in temperature and humidity-controlled quarters with free access to food and fresh tap water. They were mated with young adult males, and observation of the vaginal sperm plug was considered Day 0 of gestation. Beginning on Day 6 and continuing daily through Day 15 of gestation, the females were dosed with the indicated dosages by oral intubation; the controls were sham treated.

Body weights were recorded on Days 0, 6, 11, 15, and 20 of gestation. All animals were observed daily for appearance and behavior with particular attention to food consumption and weight, in order to rule out any abnormalities which may have occurred as a result of anorexic effects in the pregnant female animal.

On Day 20 all dams were subjected to Caesarean section under surgical anesthesia, and the numbers of implantation sites, resorption sites, and live and dead fetuses were recorded. The body weights of the live pups were also recorded. The urogenital tract of each dam was examined in detail for anatomical normality.

All fetuses were examined grossly for the presence of external congenital abnormalities. One-third of the fetuses of each litter underwent detailed visceral examinations employing 10X magnification. The remaining two-thirds were cleared in potassium hydroxide (KOH), stained with alizarin red S dye and examined for skeletal defects.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 1 | Appendix II | Date February 15, 1972

Material FDA 71-1 | Reproduction Data in Rats (Individual) | Laboratory No. 0729

Dose Sham

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
S 9001	P	10	9		1	3.81	
S 9002	P	10	10			4.11	
S 9003	NP	0				--	
S 9004	P	11	11			5.86	
S 9005	P	10	10			3.65	
S 9006	P	12	12			4.17	
S 9007	P	16	16			4.16	
S 9008	P	11	11			3.87	
S 9009	P	12	12			4.93	
S 9010	P	12	12			3.56	
S 9011	P	14	14			3.87	
S 9012	P	15	15			3.88	
S 9013	P	12	12			3.71	
S 9014	P	10	10			3.37	
S 9015	P	9	8		1	4.02	
S 9016	P	10	10			3.89	
S 9017	P	13	13			3.68	
S 9018	P	6	6			3.57	
S 9019	P	13	13			3.45	
S 9020	P	13	12		1	3.72	
S 9021	NP	0				--	
S 9022	P	14	14			6.07	
S 9023	P	12	12			3.80	
S 9024	NP	0				--	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 2

Material Aspirin

Dose 250 mg/kg

Appendix II

Reproduction Data in Rats (Individual)

Date February 15, 1972

Laboratory No. 0729

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
A 9001	NP	0				--	Died Day 16.
A 9002	NP	0				--	Died Day 19.
A 9003	P	8	8			3.52	
A 9004	NP	0				--	Died Day 15
A 9005	NP	0				--	
A 9006	P	10	10			3.29	
A 9007	P	11	11			3.37	
A 9008	P	15	14		1	3.39	
A 9009	P	10	8		2	3.49	
A 9010	P	14	14			3.13	
A 9011	P	11	11			5.13	
A 9012	P	12	11		1	3.27	
A 9013	P	16	15		1	3.48	
A 9014	NP	0				--	
A 9015	P	12	12			3.48	
A 9016	P	11		9	2	--	Died Day 19.
A 9017	P	11	11			3.70	
A 9018	P	14	11		3	3.59	
A 9019	P	13	13			3.77	
A 9020	P	12	12			3.62	
A 9021	P	13	13			3.20	
A 9022	NP	0				--	
A 9023	P	10	10			3.39	
A 9024	P	8	7		1	3.41	
A 9025	P	5	1		4	3.57	
A 9026	P	14	14			3.73	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 3

Material FDA 71-1

Dose 27 mg/kg

Appendix II

Reproduction Data in Rats (Individual)

Date February 15, 1972

Laboratory No. 0729 b

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
B 9001	P	14	14			3.79	
B 9002	P	11	11			3.64	
B 9003	NP	0				--	
B 9004	P	7	6		1	5.59	
B 9005	P	10	9		1	4.05	
B 9006	P	8	8			4.03	
B 9007	P	11	11			3.41	
B 9008	P	11	10		1	4.00	
B 9009	P	12	11		1	3.65	
B 9010	P	8	7		1	3.67	
B 9011	P	13	13			3.54	
B 9012	P	9	8		1	5.59	
B 9013	NP	0				--	
B 9014	P	11	9		2	3.83	
B 9015	P	9	8		1	4.18	
B 9016	P	12	11		1	3.86	
B 9017	P	10	10			3.35	
B 9018	P	16	16			3.37	
B 9019	P	12	12			3.81	
B 9020	P	6	3		3	3.38	
B 9021	P	12	12			4.08	
B 9022	P	1	1			6.40	
B 9023	P	12	12			3.14	
B 9024	P	5			5	--	

* P = Pregnant: NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 4

Material FDA 71-1

Dose 90 mg/kg

Appendix II

Reproduction Data in Rats (Individual)

Date February 15, 1972

Laboratory No. 0729 b

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
B 9031	P	12	10		2	3.54	
B 9032	P	17	17			3.96	
B 9033	NP	0				--	Died Day 20.
B 9034	P	11	4		7	3.75	
B 9035	NP	0				--	
B 9036	P	11	11			4.01	
B 9037	P	14	10		4	3.65	
B 9038	P	12	12			3.81	
B 9039	P	13	12		1	3.82	
B 9040	NP	0				--	
B 9041	P	9	9			4.05	
B 9042	P	11	11			3.63	
B 9043	P	7	6		1	4.15	
B 9044	P	14	13		1	3.67	
B 9045	NP	0				--	Died Day 12.
B 9046	P	13	12		1	3.63	
B 9047	P	9	9			3.96	
B 9048	P	11	11			4.13	
B 9049	P	14	14			3.93	
B 9050	NP	0				--	Died Day 14.
B 9051	P	6	6			3.57	
B 9052	NP	0				--	
B 9053	P	6	6			6.87	
B 9054	P	11	11			4.06	
B 9055	P	7	5		2	3.79	
B 9056	P	10	10			3.69	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 5 | Appendix II | Date February 15, 1972

Material FDA 71-1 | Reproduction Data in Rats (Individual) | Laboratory No. 0729 b

Dose 300 mg/kg

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
B 9061	P	11	10		1	3.46	
B 9062	NP	0				--	Died Day 17.
B 9063	P	9	9			4.36	
B 9064	NP	0				--	
B 9065	P	11	11			--	Died Day 8.
B 9066	NP	0				--	
B 9067	P	11	11			3.59	
B 9068	P	12	12			3.46	
B 9069	P	11	8	1	2	3.17	
B 9070	P	14	14			3.24	
B 9071	P	13	13			3.51	
B 9072	P	12	12			3.23	
B 9073	NP	0				--	
B 9074	P	11	11			3.80	
B 9075	P	11	8		3	3.54	
B 9076	P	11	11			3.63	
B 9077	P	14	14			4.06	
B 9078	P	10	9		1	3.72	
B 9079	P	12	12			3.55	
B 9080	P	15	15			--	Died Day 14.
B 9081	P	11	11			3.78	
B 9082	P	17	17				Died Day 8.
B 9083	P	15	15			3.94	
B 9084	P	12	12			3.28	
B 9085	P	11	11			4.21	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 6

Material FDA 71-1

Dose 1000 mg/kg

Appendix II

Reproduction Data in Rats (Individual)

Date February 15, 1972

Laboratory No. 0729 b

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
B 9091	P	12	12			3.99	
B 9092	P	15	14		1	3.79	
B 9093	P	10	10			3.75	
B 9094	P	9	9			3.94	
B 9095	NP	0				--	Died Day 10.
B 9096	P	14	14			3.86	
B 9097	P	10	10			3.80	
B 9098	P	9	7		2	3.58	
B 9099	P	10	10			3.75	
B 9100	P	15	15			3.39	
B 9101	P	12	11		1	3.77	
B 9102	P	12	12			3.55	
B 9103	NP	0				--	
B 9104	P	7	7			3.56	
B 9105	P	11	11			3.75	
B 9106	P	13	13			3.34	
B 9107	NP	0				--	Died Day 10.
B 9108	P	14	13		1	3.62	
B 9109	P	7	7			4.20	
B 9110	P	12	12			3.54	
B 9111	P	12	11		1	4.16	
B 9112	P	10		10		--	Died Day 20.
B 9113	P	13	12	1		3.70	
B 9114	P	14	14			--	Died Day 13.
B 9115	P	13	12		1	3.50	

* P = Pregnant: NP = Not Pregnant

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FINAL REPORT

Submitted to: DHEW/Public Health Service
Food and Drug Administration CA-272
5600 Fishers Lane-Room 5C-13
Rockville, Maryland 20852

Date: February 15, 1972

Laboratory No. 0730 b
Contract No. FDA 71-260

Sample: Fine dark brown powdered material.

Marking: FDA 71-1 (Ammonium glycyrrhizinate).

Examination Requested: Teratologic evaluation of FDA 71-1 in hamsters.

Procedure: See Appendix I.

Results: See Tables 1 through 4 and Appendix II.

Conclusion: Attention is called to the fact that this is the first of a series of reports which will be issued in accordance with the terms of the contract cited above. Eventually, a total of at least 36 compounds will have been tested in 18 pairs; each pair being run concurrently against one sham-treated control and one positive control group. Because of the inherent variability of biological data of the type dealt with here, the accumulation and pooling of sequential sets of control values will greatly enhance the statistical value of the findings and the ultimate reliability of the test results.

For these reasons, the conclusion stated below is regarded as provisional and subject to reexamination in the light of later findings:

"The administration of up to 1000 mg/kg (body weight) of the test material to pregnant hamsters for 5 consecutive days had no clearly discernible effect on nidation or on maternal or fetal survival. The number of abnormalities seen in either soft or skeletal tissues of the test groups did not differ from the number occurring spontaneously in the sham-treated controls."

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Kenneth Morgareidge

Kenneth Morgareidge, Ph.D.
Vice President

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups: 1 through 6

Material: FDA 71-1

Date February 15, 1972

Laboratory No. 0730 b

Table 1

Fate Summary
(Hamsters)

Group	Material	Dose mg/kg	Total		At Term	
			Mated	Pregnant	Surviving (Total)	Number Pregnant
1	Sham	0	23	22	23	21
2	Aspirin*	250	24	21	24	21
3	FDA 71-1	27	24	21	24	21
4	FDA 71-1	90	24	22	24	22
5	FDA 71-1	300	24	23	24	23
6	FDA 71-1	1000	25	23	25	23

* Positive Control

Group: 1 through 6

Material: FDA 71-1

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Table 2

Reproduction Data
(Hamsters)

Date February 15, 1972

Laboratory No. 0730 b

Group:	1	2	3	4	5	6
Dose (mg/kg):	Sham	Aspirin*	27	90	300	1000
Number of females:						
Total pregnant	22	21	21	22	23	23
Pregnant at term	21	21	21	22	23	23
Number of live litters:	21	21	21	22	23	23
Number of implant sites:						
Total	281	262	275	297	297	309
Average/pregnant dam	12.8	12.5	13.1	13.5	12.9	14.0
Number of fetuses alive:						
Total	264	257	265	287	279	298
Average/live litter	12.6	12.2	12.6	13.0	12.1	13.0
Average/pregnancy at term	12.6	12.2	12.6	13.0	12.1	13.0
Number of fetuses dead:						
Total	0	1	0	0	3	1
Litters with one or more dead	0	1	0	0	3	1
Pregnancies at term (%)	0	4.8	0	0	13.0	4.3
Litters with all dead	0	0	0	0	0	0
Pregnancies at term (%)	0	0	0	0	0	0
Number of resorptions:						
Total:	17	4	10	10	15	10
Litters with one or more resorptions	5	3	7	5	9	9
Pregnancies at term (%)	23.8	14.3	33.3	22.7	39.1	39.1
Litters with total resorptions	1	0	0	0	0	0
Pregnancies at term (%)	4.8	0	0	0	0	0
Average fetus weight, g.	1.72	1.81	1.87	1.80	1.80	1.80

* Positive control at 250 mg/kg

Groups 1 ough 6

Material FDA 71-1

ble 3

Summary of Skeletal Findings
(Hamsters)

Laborato o. 0730

Date February 15, 197

Findings	Group No:	1	2	3	4	5	6
	Dose (mg/kg):	Sham	Aspirin**	27	90	300	1000
Live Fetuses Examined		189/21	184/21	192/21	202/22	207/23	213/23
Fetuses with:							
Incomplete sternebrae		17/6	20/10	27/13	35/13	28/17	19/7
Scrambled sternebrae							
Bipartite sternebrae		115/19	91/19	122/21	143/21	123/19	150/22
Missing sternebrae							
Fused sternebrae					1/1		
Extra sternebrae				1/1			
Incomplete hyoid				1/1	8/5		3/3
Fetuses with:							
Scrambled vertebrae							
Tail defects (short, scrambled, etc.)							
Incomplete ossification: Vertebrae		54/12	82/17	92/18	91/18	90/18	97/16
Fetuses with:							
Fused ribs		1/1				1/1	
Incomplete ribs		5/4	1/1		26/14	3/3	6/5
Wavy ribs							
Less than 12 ribs							
More than 13 ribs			1/1		16/7	15/8	10/7
Other Findings:							
Scoliosis							
Delayed cranial ossification		17/2	10/2	1/1	19/8	23/6	100/14
Craniostosis					1/1	1/1	
Feet; retarded ossification		9/2	1/1	6/5		27/4	6/2

*Numerator = Number of fetuses affected; Denominator = number of litters affected

** Positive control at 250 mg/kg

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 1 through 6

Species Hamsters

Date February 15, 1972

Laboratory No. 0730 b

Table 4

Average Body Weights *

Group	Material	Dose Level	Day 0	6	8	10	14**
		mg/kg	g				
1	Sham	0	106.2	110.2	113.7	124.7	144.8 (21)
2	Aspirin	250	104.9	109.3	112.6	121.8	144.8 (21)
3	FDA 71-1	27	110.6	116.4	120.1	132.4	153.5 (21)
4	FDA 71-1	90	111.0	115.5	118.1	131.4	153.8 (22)
5	FDA 71-1	300	109.7	113.3	116.3	127.7	149.2 (23)
6	FDA 71-1	1000	110.7	115.3	119.9	131.6	154.3 (23)

* of pregnant dams
** Number of surviving dams in parentheses (c.f. Table 1).

Appendix I

Teratology Study in Hamsters

Virgin adult female golden hamsters from an outbred strain were individually housed in mesh bottom cages in temperature and humidity controlled quarters with free access to food and fresh tap water at all times. They were mated (1 to 1) with mature males and the appearance of motile sperm in the vaginal smear was considered as Day 0 of gestation. Beginning on Day 6 and continuing daily through Day 10 of gestation, the indicated dose levels of the test material were administered by oral intubation; the controls were sham-treated.

Body weights were recorded on Days 0, 8, 10, and 14 of the gestation period. All animals were observed daily for appearance and behavior with particular attention to food consumption in order to better recognize any abnormalities resulting from anorexic effects in the pregnant animal.

On Day 15, all animals were subjected to Caesarian section under deep anesthesia and the numbers of implantation sites, resorption sites, live and dead fetuses were recorded. All live pups were weighed and the genital tract of each dam was examined for any anatomical abnormalities.

All fetuses were examined grossly for the presence of external congenital defects and one-third of each litter underwent detailed visceral examination under 10X magnification. The remaining two-thirds of the pups were cleared in potassium hydroxide, stained with alizarin red dye, and examined for the presence of sketal abnormalities.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 1 | Appendix II | Date February 15, 1972

Material Sham | Reproduction Data in Hamsters (Individual) | Laboratory No. 0730

Dose 0

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
S 0001	P	12	12			1.62	
S 0002	P	12	12			1.82	
S 0003	P	9	9			1.66	
S 0004	P	14	14			2.01	
S 0005	P	17	15		2	1.97	
S 0006	P	14	14			2.06	
S 0007	P	10	10			1.94	
S 0008							Number not assigned.
S 0009	P	14	12		2	1.85	
S 0010	P	12	12			1.03	
S 0011	P	13	13			1.78	
S 0012	P	16	15		1	1.63	
S 0013	P	14	13		1	1.88	
S 0014	P	14	14			1.80	
S 0015	P	17	17			2.00	
S 0016	P	10	10			0.98	
S 0017	P	15	15			1.70	
S 0018	P	10	10			1.65	
S 0019	P	16	16			1.84	
S 0020	P	12	12			1.68	
S 0021	NP	0				--	
S 0022	P	10	10			1.77	
S 0023	P	9	9			1.55	
S 0024	P	11			11	--	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 2 | Appendix II | Date February 15, 1972

Material Aspirin | Reproduction Data in Hamsters (Individual) | Laboratory No. 0730

Dose 250 mg/kg

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
A 0001	P	13	13			1.75	
A 0002	NP	0				--	
A 0003	P	13	13			1.82	
A 0004	P	12	11	1		2.12	
A 0005	P	12	12			1.90	
A 0006	NP	0				--	
A 0007	P	15	15			2.10	
A 0008	NP	0				--	
A 0009	P	13	13			1.67	
A 0010	P	12	12			1.04	
A 0011	P	14	14			1.91	
A 0012	P	11	11			1.67	
A 0013	P	11	10		1	1.94	
A 0014	P	14	13		1	1.82	
A 0015	P	16	16			1.80	
A 0016	P	12	12			1.61	
A 0017	P	13	13			1.89	
A 0018	P	13	13			1.87	
A 0019	P	12	12			1.74	
A 0020	P	12	12			1.84	
A 0021	P	14	14			1.62	
A 0022	P	11	11			1.69	
A 0023	P	10	8		2	1.86	
A 0024	P	9	9			2.42	

* P = Pregnant: NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 3 | Appendix II | Date February 15, 1972

Material FDA 71-1 | Reproduction Data in Hamsters (Individual) | Laboratory No. 0730 b

Dose 27 mg/kg

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
B 0001	P	13	13			1.69	
B 0002	P	12	12			1.93	
B 0003	P	16	16			1.94	
B 0004	P	12	11		1	1.94	
B 0005	NP	0				--	
B 0006	P	13	13			1.99	
B 0007	P	13	13			1.85	
B 0008	P	14	14			1.91	
B 0009	P	11	11			2.83	
B 0010	P	9	8		1	1.45	
B 0011	P	12	11		1	1.82	
B 0012	NP	0				--	
B 0013	P	15	15			1.78	
B 0014	P	14	14			1.97	
B 0015	P	15	15			2.00	
B 0016	P	12	10		2	1.96	
B 0017	P	14	13		1	1.81	
B 0018	P	13	13			1.94	
B 0019	NP	0				--	
B 0020	P	13	13			1.56	
B 0021	P	14	13		1	1.85	
B 0022	P	12	12			1.78	
B 0023	P	13	13			1.72	
B 0024	P	15	12		3	1.49	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 4 | Appendix II | Date February 15, 1972

Material FDA 71-1 | Reproduction Data in Hamsters (Individual) | Laboratory No. 0730 b

Dose 90 mg/kg

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
B 0031	P	12	12			1.71	
B 0032	P	13	13			1.98	
B 0033	P	12	12			1.86	
B 0034	P	16	16			1.70	
B 0035	NP	0				--	
B 0036	NP	0				--	
B 0037	P	9	9			2.05	
B 0038	P	19	17		2	1.93	
B 0039	P	14	13		1	1.61	
B 0040	P	14	14			2.07	
B 0041	P	15	14		1	1.70	
B 0042	P	16	16			1.82	
B 0043	P	12	12			1.78	
B 0044	P	11	11			1.82	
B 0045	P	13	9		4	1.80	
B 0046	P	15	15			1.84	
B 0047	P	17	17			1.70	
B 0048	P	13	13			1.87	
B 0049	P	12	12			1.80	
B 0050	P	11	11			1.85	
B 0051	P	12	12			1.72	
B 0052	P	13	13			1.48	
B 0053	P	14	14			1.62	
B 0054	P	14	12		2	1.96	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 5 | Appendix II | Date February 15, 1972

Material FDA 71-1 | Reproduction Data in Hamsters (Individual) | Laboratory No. 0730 b

Dose 300 mg/kg

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
B 0061	P	12	12			1.92	
B 0062	P	12	12			1.92	
B 0063	P	17	14		3	1.77	
B 0064	P	16	16			1.92	
B 0065	P	15	15			1.82	
B 0066	P	9	8		1	1.87	
B 0067	P	12	12			1.77	
B 0068	P	12	11		1	1.90	
B 0069	P	11	11			1.08	
B 0070	P	17	14		3	1.80	
B 0071	P	13	13			2.08	
B 0072	P	9	7	1	1	1.82	
B 0073	P	11	11			1.81	
B 0074	P	16	16			2.00	
B 0075	P	13	12	1		1.72	
B 0076	P	15	15			1.55	
B 0077	P	11	10	1		1.81	
B 0078	P	13	13			2.01	
B 0079	P	10	9		1	1.63	
B 0080	P	16	15		1	1.74	
B 0081	P	12	9		3	1.89	
B 0082	P	14	13		1	1.83	
B 0083	NP	0				--	
B 0084	P	11	11			1.70	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 6 | Appendix II | Date February 15,1972

Material FDA 71-1 | Reproduction Data in Hamsters (Individual) | Laboratory No. 0730 b

Dose 1000 mg/kg

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
B 0091	P	12	11		1	1.92	
B 0092	P	13	13			2.08	
B 0093	P	15	15			2.02	
B 0094	P	11	10		1	1.55	
B 0095	P	13	13			1.98	
B 0096	P	12	12			1.98	
B 0097	P	16	16			1.56	
B 0098	P	16	16			1.86	
B 0099	P	14	13		1	0.89	
B 0100	P	15	15			2.04	
B 0101	NP	0				--	
B 0102	P	12	12			1.73	
B 0103	P	14	14			1.77	
B 0104	P	12	11		1	1.94	
B 0105	P	15	13		2	1.71	
B 0106	P	12	12			1.72	
B 0107	P	16	15	1		1.75	
B 0108	P	13	13			1.94	
B 0109	P	14	14			1.94	
B 0110	P	15	14		1	1.79	
B 0111	P	13	12		1	1.72	
B 0112	P	11	10		1	1.79	
B 0113	NP	0				--	
B 0114	P	14	13		1	1.74	
B 0115	P	11	11			1.88	

* P = Pregnant: NP = Not Pregnant

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FINAL REPORT

Submitted to: DHEW/Public Health Service
Food and Drug Administration CA-272
5600 Fishers Lane-Room 5C-13
Rockville, Maryland 20852

Date: February 15, 1972

Laboratory No. 0731 b
Contract No. FDA 71-260

Sample: Fine dark brown powdered material.

Marking: FDA 71-1 (Ammonium glycyrrhizinate).

Examination Requested: Teratologic evaluation of FDA 71-1 in rabbits.

Procedure: See Appendix I.

Results: See Tables 1 through 4 and Appendix II.

Conclusion: Attention is called to the fact that this is the first of a series of reports which will be issued in accordance with the terms of the contract cited above. Eventually, a total of at least 36 compounds will have been tested in 18 pairs; each pair being run concurrently against one sham-treated control and one positive control group. Because of the inherent variability of biological data of the type dealt with here, the accumulation and pooling of sequential sets of control values will greatly enhance the statistical value of the findings and the ultimate reliability of the test results.

For these reasons, the conclusion stated below is regarded as provisional and subject to reexamination in the light of later findings:

"The administration of up to 1000 mg/kg (body weight) of the test material to pregnant rabbits for 13 consecutive days had no clearly discernible effect on nidation or on maternal or fetal survival. The number of abnormalities seen in either soft or skeletal tissues of the test groups did not differ from the number occurring spontaneously in the sham-treated controls."

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Kenneth Morgareidge

Kenneth Morgareidge, Ph.D.
Vice President

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups: 1 through 6

Material: FDA 71-1

Date February 15, 1972

Laboratory No. 0731 b

Table 1

Fate Summary
(Rabbits)

Group	Material	Dose mg/kg	Total		At Term	
			Mated	Pregnant	Surviving (Total)	Number Pregnant
1	Sham	0	15	10	13	8
2	6-AN *	5	15	10	14	4
3	FDA 71-1	27	15	12	15	12
4	FDA 71-1	90	15	10	15	10
5	FDA 71-1	300	15	11	14	10
6	FDA 71-1	1000	15	10	10	7

* Positive Control: 6 - Amino nicotinamide dosed on Day 9.

Group: [illegible]rough 6

Material: FDA 71-1

Tabl[illegible]2

Reproduction Data

(Rabbits)

Date February 15, [illegible]2

Laboratory No. 0731 b

Group:	1	2	3	4	5	6
Dose (mg/kg):	Sham	6-AN*	27	90	300	1000
Number of females:						
Total pregnant	10	10	12	10	11	10
Pregnant at term	8	4	12	10	10	7
Number of corpora lutea:						
Total	106	87	111	89	96	75
Average/pregnant dam	10.6	8.7	9.25	8.9	8.7	7.5
Number of live litters:	6	4	12	10	10	7
Number of implant sites:						
Total	39	30	70	52	56	46
Average/pregnant dam	3.9	3.0	5.8	5.2	5.1	4.6
Number of fetuses alive:						
Total	14	8	64	42	48	38
Average/pregnant dam	1.40	0.80	5.3	4.2	4.4	3.8
Average/live litter	2.33	2.0	5.3	4.2	4.8	5.4
Average/pregnancy at term	1.75	2.0	5.3	4.2	4.8	5.4
Number of fetuses dead:						
Total	12	1	0	5	0	6
Litters with one or more dead	3	1	-	2	-	2
Pregnancies at term (%)	37.5	25.0	-	40.0	-	28.5
Litters with all dead	2	0	-	1	-	1
Pregnancies at term (%)	25.0	-	-	10.0	-	14.2
Number of resorptions:						
Total:	13	25	6	5	8	2
Litters with one or more resorptions	7	9	6	4	3	1
Pregnancies at term (%)	87.5	>100	50.0	40.0	30.0	14.2
Litters with total resorptions	2	6	0	0	1	0
Pregnancies at term (%)	25.0	>100	-	-	10.0	-
Average fetus weight, g.	40.5	30.0	34.9	34.9	40.3	39.4

* Positive control: 5.0 mg/kg 6 amino nicotinamide dosed on Day 9.

Groups [illegible] through 6

Material FDA 71-1

[illegible]ble 3

Summary of Skeletal Findings
(Rabbits)

Laborat[illegible] No. 0731 [illegible]

Date February 15, 19[illegible]

Findings	Group No:	1	2	3	4	5	6
	Dose (mg/kg):	Sham	6-AN**	27	90	300	1000
Live Fetuses Examined		14/6	8/4	64/12	45/10	48/10	34/7
Fetuses with:							
Incomplete sternebrae		7/3	4/3	25/10	20/7	12/6	4/3
Scrambled sternebrae			2/2	2/2	1/1	1/1	2/2
Bipartite sternebrae			2/2			1/1	
Missing sternebrae			1/1	1/1	1/1		
Fused sternebrae			2/2	6/4		1/1	3/2
Extra sternebrae							
Fetuses with:							
Scrambled vertebrae							
Tail defects (short, scrambled, etc.)							
Club foot			2/2				
Fetuses with:							
Fused ribs			2/2				
Incomplete ribs		2/2	2/2	4/2	8/5	10/6	1/1
Wavy ribs				1/1			
Less than 12			1/1				
Other Findings:							
Scoliosis							
Delayed cranial ossification							
Craniostosis		6/3	4/2	15/7	5/5	8/4	
Craniobifida				1/1			

*Numerator = Number of fetuses affected; Denominator = number of litters affected

** Positive control at 5 mg/kg of 6-amino nicotinamide on day 9.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 1 through 6

Species Rabbits

Date February 15, 1972

Laboratory No. 0731 b

Table 4

Average Body Weights *

Group	Material	Dose Level	Day 0	6	12	18	29**
		mg/kg	kg				
1	Sham	0	2.39	2.39	2.36	2.31	2.31 (8)
2	6-AN***	5	2.34	2.43	2.27	----	2.09 (9)
3	FDA 71-1	27	2.41	2.41	2.40	2.32	2.40 (12)
4	FDA 71-1	90	2.52	2.53	2.51	2.51	2.49 (10)
5	FDA 71-1	300	2.34	2.35	2.34	2.33	2.34 (10)
6	FDA 71-1	1000	2.26	2.38	2.33	2.23	2.60 (7)

* Of pregnant dams.
** Number of surviving dams in parentheses (c.f. Table 1).
*** 6-amino nicotinamide dosed on Day 9.

Appendix I

Teratology Study in Rabbits

Virgin, adult, Dutch-belted female rabbits were individually housed in mesh bottom cages in temperature and humidity-controlled quarters with free access to food and fresh tap water. On Day 0, each doe was given an injection of 0.4 ml of human chorionic gonadotropin (400 IU) via the marginal ear vein. Three hours later, each doe was inseminated artificially with 0.3 ml of diluted semen from a proven donor buck using approximately 20×10^6 motile sperm according to the procedure described by Vogin et al (Pharmacologist 11, 282 (1969)). Beginning on Day 6 and continuing daily through Day 18 the females were dosed with the indicated dosages by oral intubation; the controls were sham treated.

Body weights were recorded on Days 0, 6, 12, 18, and 29 of gestation. All animals were observed daily for appearance and behavior, with particular attention to food consumption and body weight in order to rule out any abnormalities which may have occurred as a result of anorexic effects in the pregnant female animal.

On Day 29 all does were subjected to Caesarean section under surgical anesthesia, and the numbers of corpora lutea, implantation sites, resorption sites and live and dead fetuses were recorded. Body weights of the live pups were also recorded. The urogenital tract of each animal was examined in detail for normality. In addition all fetuses underwent a detailed gross examination for the presence of external congenital abnormalities. The live fetuses of

each litter were then placed in an incubator for 24 hours for the evaluation of neonatal survival. All surviving pups were sacrificed, and all pups examined for visceral abnormalities (by dissection). All fetuses were then cleared in potassium hydroxide (KOH), stained with alizarin red S dye and examined for skeletal defects.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 1 | Appendix II | Date February 15, 1972

Material Sham | Reproduction Data in Rabbits (Individual) | Laboratory No. 0731

Dose 0

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
S 1001	P	13	3	3			38.4	
S 1002	P	6	2	1		1	36.1	
S 1003	NP	0	0				---	
S 1004	NP	4	0				---	
S 1005	P	9	6	5		1	40.3	
S 1006	P	13	3	2		1	43.9	
S 1007	P	7	7		7		---	Died day 20.
S 1008	NP	0	0				---	
S 1009	P	5	4		4		---	Died day 10.
S 1010	NP	0	0				---	
S 1011	P	4	2			2	---	
S 1012	P	17	5	1	1	3	40.7	
S 1013	P	13	4	2		2	43.7	
S 1014	P	5	3			3	---	
S 1015	NP	10	0				---	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Appendix II

Reproduction Data in Rabbits (Individual)

Group 2

Material 6- Amino nicotinamide

Dose 5 mg/kg

Date February 15, 1972

Laboratory No. 0731

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
Z 1001	P	2	2			2	---	
Z 1002	P	5	2			2	---	
Z 1003	P	5	4			4	---	
Z 1004	P	3	1			1	---	
Z 1005	P	9	5	1	1	3	24.1	1 Neonatal death.
Z 1006	NP	4	0				---	
Z 1007	P	10	2	1		1	20.1	1 Neonatal death.
Z 1008	P	6	4			4	---	
Z 1009	NP	5	0				---	
Z 1010	NP	4	0				---	
Z 1011	P	4	0			4	---	
Z 1012	P	12	4	4			38.6	
Z 1013	P	10	6	2		4	37.3	2 Neonatal deaths.
Z 1014	NP	5	0				---	Died day 22.
Z 1015	NP	3	0				---	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 3

Appendix II

Date February 15, 1972

Material FDA 71-1

Reproduction Data in Rabbits (Individual)

Laboratory No. 0731 b

Dose 27 mg/kg

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
B 1001	P	6	4	4			32.4	4 Neonatal deaths.
B 1002	P	7	6	6			38.1	1 Neonatal death.
B 1003	P	10	4	3		1	38.7	
B 1004	P	13	8	8			26.3	
B 1005	P	10	6	6			38.5	
B 1006	P	9	4	3		1	34.9	
B 1007	P	8	8	8			31.6	1 Neonatal death.
B 1008	P	11	7	6		1	32.4	
B 1009	P	8	6	6			28.8	
B 1010	P	4	2	1		1	42.1	
B 1011	P	8	8	7		1	32.1	1 Neonatal death.
B 1012	NP	0	0				---	
B 1013	NP	3	0				---	Pyometra.
B 1014	NP	0	0				---	
B 1015	P	14	7	6		1	43.3	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Appendix II

Reproduction Data in Rabbits (Individual)

Group 4

Material FDA 71-1

Dose 90 mg/kg

Date February 15, 1972

Laboratory No. 0731 b

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
B 1016	P	12	9	9			27.0	
B 1017	P	5	5	5			40.6	5 Neonatal deaths.
B 1018	P	4	4	4			44.6	
B 1019	P	12	8	5	1	2	30.7	
B 1020	P	7	5		4	1	30.5	
B 1021	NP	0	0				---	
B 1022	NP	8	0				---	
B 1023	P	5	4	3		1	39.3	
B 1024	NP	0	0				---	No ovaries.
B 1025	P	7	7	7			34.4	
B 1026	NP	0	0				---	
B 1027	P	8	6	6			34.9	
B 1028	P	13	1	1			33.3	
B 1029	P	8	3	2		1	34.1	
B 1030	NP	0	0				---	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 5 | Appendix II | Date February 15, 1972

Material FDA 71-1 | Reproduction Data in Rabbits (Individual) | Laboratory No. 0731 b

Dose 300 mg/kg

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
B 1031	NP	0	0				---	
B 1032	P	11	5	5			42.8	
B 1033	NP	0	0				---	
B 1034	P	7	6	6			42.5	
B 1035	P	17	4	4			41.0	
B 1036	P	7	4	4			37.7	
B 1037	NP	0	0				---	
B 1038	P	6	3	3			42.3	
B 1039	P	6	2	2			42.0	
B 1040	P	11	7	6		1	33.8	
B 1041	P	10	8	8			41.2	1 Neonatal death.
B 1042	NP	0	0				---	
B 1043	P	7	6			6	---	Aborted Day 28.
B 1044	P	4	4	3		1	37.7	
B 1045	P	10	7	7			42.1	1 Neonatal death.

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 6

Appendix II

Date February 15, 1972

Material FDA 71-1

Reproduction Data in Rabbits (Individual)

Laboratory No. 0731 b

Dose 1000 mg/kg

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Resorption Sites	Average Fetus Weight (g)	Remarks
B 1046	NP	0	0	-			---	
B 1047	NP	0	0				---	
B 1048	P	10	2	2			54.6	1 Neonatal death.
B 1049	P	8	7	7			45.0	
B 1050	P	10	10	10			36.1	
B 1051	NP	0	0				---	
B 1052	P	7	2	2			23.2	Died Day 19.
B 1053	P	6	3	3			---	Died Day 18; Dosing mishap
B 1054	P	7	3		1	2	---	Aborted Day 21.
B 1055	P	5	5		5		---	Aborted Day 22.
B 1056	NP	0	0				---	Died Day 26.
B 1057	NP	0	0				---	
B 1058	P	10	9	9			29.9	
B 1059	P	7	2	2			40.0	
B 1060	P	5	3	3			47.4	

* P = Pregnant; NP = Not Pregnant

Rev. Lyonn. Med.
Nov. 1968, pp.785-790

Does Glycyrrhizin act by Adrenocortical Stimulation? Biological Simulation of Conn's Syndrome by Glycyrrhizin Intoxication.

by

A. Froment, J. Sassard, Ch. Bizollon and M. Vincent*

* Work of Research Unit for the Study of Atherosclerosis and Arterial Hypertension. I.N.S.E.R.M., 22 Avenue du Doyen Lepine, 69 Lyon-Bron.

The case which is the subject of the present work has been briefly referred to in an earlier work (4, see p. 645). At the same time J. Tourniaire et al. (9) have reported a case of glycyrrhizin intoxication with hyperaldosteronism, in which they were able to demonstrate the chronological relationship between the consumption of the toxic substance and the increase in aldosteronuia. Thus the problem previously dealt with experimentally arose on the clinical plane, namely the stimulation of the adrenal cortex by glycyrrhizin. The following observations would seem to furnish a few arguments in support of this interpretation.

Case: TO... Gaston, (N: H 11.618).

Thirty five year old man hospitalized for arterial hypertension and arterial obliteration of the lower limbs. Because of consumption of considerable quantities of glycyrrhizin, laboratory tests were made which revealed increased aldosteronuria

during consumption of the toxic substance, with zero plasma renin activity. Discontinuation of intoxication was followed by considerable loss of sodium. 8 weeks after cessation of ingestion of the toxic substance, with no other therapeutic measures, the renin activity and aldosteronuria were normal, while arterial blood pressure remained high.

This 35 year old man underwent a disintoxication cure for alcoholism in 1963. He recalled that his hypertension was discovered in 1966, but believes that at the age of 15 or 16 an increased blood pressure had been discovered of 160 mm Hg. For the past year he complained of intermittent lameness of the left leg. He was hospitalized on Nov. 2, 1967 after a blood pressure of 230/125 was established.

Hospital examination showed:

A fairly severe rise in blood pressure: average value during the first 6 days 193/103 mm Hg;

No clinical or radiological signs of cardiac implications. On the other hand, the EKG showed signs of left ventricular hypertrophy (SV2 + RV5 = 50 mm) with a horizontal axis. Repolarization was normal, but there was a picture of possible necrosis in previous derivations, but no prior history.

The fundus of the eye showed moderately slender and irregular arteries, without notable anomalies in coloration and without Gunn's crossing sign. There was no indication of malignant retinopathy.

Renal function was normal:

Proteinuria = 0

Blood urea between 0.14 and 0.48 g/l in different determinations.

Serum creatinine = 9 mg/l

Endogenous creatinine clearance = 108 ml/mn/1.73 m^2

The following data were also noted:

Weight = 72.5 kg, height 1.81 m

Cholesterol = 2.42 g/l

Triglycerides = 0.91 g/l

Fasting glycemia = 0.90 g/l.

An etiolgogic evaluation of the hypertension was carried out.

Urography IV was strictly normal.

Renal arteriography revealed a non-stenosing atheromatic plaque at the point of origin of the right renal artery. There was an incomplete stenosis of the left and right external iliac.

The Addis count (red cells and leukocytes) was normal.

The vanyl-mandelic acid elimination was normal (6.5 mg/day), and urinary catecholamine determination on de-medullated dogs was negative.

Before hospitalization the etiologic search was guided by the discovery in the course of systematic questioning of glycyrrhizin consumption in the form of Antesite following the alcoholic disintoxication cure. Antesite consumption, enhanced by constitutional polydipsia, reached 3 to 5 l/day. The glycyrrhizin dosage was found to be 34 g/month, or 1 g/day.

However, there was nothing to allow definite connection of the hypertension with the consumption of Antesite:

No asthenia, no tetanic symptoms, no Trousseau's sign, normal reflexes.

Absence of demonstrable changes in ionograms carried out before discontinuation of intoxication:

	Alk. Res. (mEq/l)	Serum: Na (mEq)	Serum: K (mEq/l)
10/16/67	4.0	149	23.0
11/3/67	3.8	144	25.8

Therefore the tests shown on Fig. 1 were carried out:

1. The consumption of Antesite was followed, consumed in the usual doses for 24 hours, to measure aldosteronuria under glycyrrhizin (method of determination by isotope dilution and double tagging with C^{14} and H^3) and measurement of renin activity of the plasma (Boucher's method).

Aldosteronuria = 20 µg/24 hours

Renin activity = 0 ng/l/min.

2. The same measurements were repeated 5 days after discontinuation of intoxication:

Aldosteronuria = 30 µg/24 hours

Renin activity = 5.0 ng/l/min.

3. Upon admission the patient's Na and K intake and loss was determined. From the 6th day he was placed on a low-sodium diet, so as to compare the sodium balance with that of patients with essential hypertension studied in the same manner. The following was observed:

Loss of sodium reaching - 419 mEq before the administration of any natriuretic;

Very slight potassium retention: + 18.5 mEq during the same period.

Parallel with this the blood pressure decreased progressively to around 145/95 mm Hg.

The administration of 25 mg/day Clorexolone from Nov. 17, 1967 did not give rise to any clearcut change in blood pressure. The cumulative balance since the cessation of intoxication, found upon release of the patient on Nov.22, 1967 was -549 mEq Na and 32.0 mEq K.

The patient was requested to interrupt the consumption of Antesite and to adhere to no special diet or take any medication.

He was seen again on Dec. 21, 1967:

Blood pressure = 190/100

Fundus of the eye = unchanged.

EKG unchanged save for a slight reduction of Sokolow's index (SV2 + RV5 = 39 mm).

No proteinuria.

Blood ionogram: K = 4.9, Na = 146, Alk. Res. - 24.0 (mEq/l).

On Janulary 15, 1968 the patient was admitted once more for additional determinations:

	K	NA	Alk. Res.
Blood Ionogr. (mEq/l)	5	144	23
Urinary ionogr. (mEq/l)	52	144	23

Aldosteronuria = 4 µg/day

Renin activity = 4.9 ng/l/min.

Since this date the patient was placed on anti-hypertension treatment (sodium restriction, natriuretic medication), but he has not been seen again in our service.

Discussion

This case presents two unusual problems:

The relationship between the arterial hypertension and the glycyrrhizin intoxication,

the explanation of the disturbances in the renin-aldosterin system.

A. So far as the demonstration of a causal link between the glycyrrhizin intoxication and the arterial hypertension, the elements of discussion were furnished in previous publications (3,4,5). In the present case the various arguments were as follows:

The dosage reached 1 g/day glycyrrhizin. It thus corresponded to the consumption observed in several subjects with a proven glycyrrhizin hypertension.

The duration of consumption was of course amply sufficient to allow such a diagnosis.

However, neither of these two arguments has any positive value in view of the different sensitivity to the toxic substance.

Polydipsia was initially considered an important positive element. While this may be the case when it appears following intoxication, the situation is obviously not the same when it precedes the intoxication and is responsible for it.

The absence of a symptomatology suggesting potassium depletion, the absence of tetanic symptoms or of any changes in the blood ionogram in no case allowed exclusion of intoxication.

The progressive reduction of blood pressure during hospitalization, while indisputable, did not end in a genuine normalization of the blood pressure at the end of the hospitalization, a feature which is difficult to evaluate.

The cumulative sodium depletion (419 mEq) significantly exceeded that of a group of persons with essential hypertension under the same conditions (129 ± 65 mEq). It did not, however, reach the values observed in our preceding cases and could have been enhanced in the present case by the pronounced polyuria.

We found it initially impossible to make a decision in favor or against glycyrrhizin intoxication. Two additional data secondarily allowed a diagnosis of arterial hypertension with concomittant glycyrrhizin consumption:

On the one hand we were aware of the blood pressure values previously found : In 1960 at the age of 28 - 3 years before any consumption of glycyrrhizin - there was a clearly abnormal elevation of blood pressure for the subject' age (160/90), which was slightly modified during the first years of intoxication (170/60 in 1963 and 1965).

On the other hand, 50 days after cessation of intoxication the blood pressure still was 190/100, while in the cases of glycyrrhizin hypertension reported so far, it always reverted to normal within this period.

These two concordant observations formally established the existence of an element of essential hypertension, independent from, pre-existing and persisting beyond intoxication.

B. The measurements of aldosteronuria and plasma renin activity carried out in the same patient gave unexpected results shown in Fig. 2:

During glycyrrhizin consumption aldosteronuria was at the upper limits of normal and there was zero plasma renin activity.

Five days after cessation of intoxication, the elevated aldosteronuria persisted, while the plasma renin activity was within the limits of normal.

Eight weeks later, aldosteronuria was strictly normal, or lowered, while the renin activity also remained within the lower limits of normal.

In view of this contrast during the consumption of glycyrrhizin between elevated aldosteronuria and zero plasma renin acitivity, it has to be linked to glycyrrhizin intoxication, since it disappeared after cessation of glycyrrhizin. In principle the same contrast is characteristic of primary hyperaldosteronism; the rare syndromes that simulate this condition (11) are either linked to an excess of potassium or to a deficiency in the synthesis of cortical hormones resulting in ACTH hyperproduction. These syndromes all have in common the stimulation of aldosterone production independently from the renin-angiotensin system, and sodium retention, which inhibits the liberation of renin without at the same time inhibiting the liberation of aldosterone. There is no other possible explanation for this biological picture.

In our case two interpretations are possible:

1. The aldosteronuria determination could be falsified by the ingestion of glycyrrhizin, due to the steroid nature of glycyrrhetinic acid. Parallel with this, this substance - independently from the adrenals - gives rise to sodium retention which inhibits the renin-aldosterone system.

Tourniaire et al. (9) have discussed and rejected this concept, notably because of the great specificity of the method of aldosterone determination used here, and the absence of elevation of aldosteronuria in a normal subject subjected to voluntary intoxication.

2. In the other version it is assumed that glycyrrhizin gives rise to inappropriate aldosterone secretion - by a mechanism which still remains to be discovered - and thus causes sodium retention and inhibition of renin secretion. This would presumably be a secondary hyperaldosteronism independent from the renin-angiotensin system, comparable, for instance, to that induced by a 17-alpha-hydroxylase deficiency.

This observation as well as that of Tourniaire et al. are not the only arguments in favor of an adrenal action of glycyrrhizin. On the contrary, other authors have already made reference to it.

In subjects with Addison's disease, glycyrrhizin is believed to be capable of replacing DOC (1,2,3). In other cases , however, it is inactive (1), sometimes after an initial phase of effectiveness, in such cases revealing a phenomenon of exhaustion (in the latter case the effectiveness tan be restored by the addition of cortisone).

The administration of spironolactone inhibits sodium retention and the loss of potassium induced by the consumption of glycyrrhizin (8).

Experimentally the administration of glycyrrhizin to adrenalectomized rats changed neither the elimination of sodium, nor the serum concentration of Na^+ and K^+ (7). Similarly, under these conditions it proves to be incapable of improving the survival of adrenalectomized animals, unlike DOC (3,7).

Sasano et al. (9) have observed that glycyrrhizin inhibits the adrenal atrophy induced by the administration of dexamethasone in rats. The effect was comparable, although to a reduced extent, to that of ACTH, and the effect appeared to be particularly distinct histologically in the fascicular and reticular tissues. The glycyrrhizin dosage used in this experimentation was extremely low (1 mg/kg/day).

While these arguments support this hypothesis, it should also be noted that they do not appear to explain all the facts, since aldosteronuria can be lowered and not elevated during the administration of glycyrrhizin (10), even when the subject shows signs of glycyrrhizin intoxication (8). It is thus possible that the mechanism considered is not constant and the variation could very well adjust to the variations in individual sensitivity to glycyrrhizin.

C. While in the case reported above we were able to conclude the coexistence of essential hypertenion and glycyrrhizin consumption independently from one another on the basis of clinical and developmental data, the laboratory findings force

us to vary this interpretation slightly, since they show that glycyrrhizin reproduces a picture of Conn's syndrome. Under these conditions it is impossible to claim that it was not involved to some degree in the relatively rapid aggravation of the arterial hypertension.

Summary

The authors report a case of a 35 year old man presenting hypertension and the consumption of large quantities of glycyrrhizin. The pre-existence of the hypertension before the intoxication, and the persistence of hypertension after cessation of the consumption of the substance lead to a diagnosis of essential hypertension. However, laboratory tests during intoxication have shown the presence of elevated aldosteronuria, while the plasma renin activity was zero. Ten weeks after the cessation of intoxication, the aldosteronuria was normal, as was the plasma renin activity. These facts as well as various data from the literature, makes it necessary to entertain the possibility of aldosterone secretion under the influence of glycyrrhizin.

Figures

1. Development of sodium and potassium values during hospitalization, as well as weight and blood pressure. The aldosteronuria values per 24 hours, the plasma renin activity, serum potassium and serum sodium values are shown on the corresponding days. Key: 1. g/day. 2. Case Tou., G. 35 years old. 3. Sodium intake. 4. Sodium-potassium balance per day. 5. Plasma renin activity. 6. serum K and Na. 7. Weight. 8. Blood pressure. 9. Days.

2. Aldosteronuria values per 24 hours and plasma renin activity during hospitalization and during control examination. The contrast between the initially high aldosteronuria and low plasma renin activity disappears 2 months after cessation of intoxication. Key. 1. ug/day. 2. Plasma renin activity.

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[illegible] BRANCH
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[illegible]

TEST ON ACUTE TOXICITY OF
CRUDE AMMONIUM GLYCYRRHIZINATE,
CRUDE POTASSIUM GLYCYRRHIZINATE,
AND MONO-AMMONIUM GLYCYRRHIZINATE.

MARUZEN PHARMACEUTICAL CO. LTD

TEST ON ACUTE TOXICITY OF CRUDE AMMONIUM GLYCYRRHIZINATE, CRUDE POTASSIUM GLYCYRRHIZINATE, AND MONO-AMMONIUM GLYCYRRHIZINATE.

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TEST MATERIALS

(Products of Maruzen Pharmaceutical Company)

Crude Ammonium Glycyrrhizinate (CAG)
Crude Potassium Glycyrrhizinate (CPG)
Mono-ammonium Glycyrrhizinate (MAG)

TEST RESULTS

The groups of six mice regardless of sex weighing from 14 to 17 g. were used for each material being tested. One to 10% aqueous solution of each material was administered to each mouse intraperitoneally or orally.

The animals were then kept until 48 hours later and observed for general conditions and mortality. From the result obtained the LD_{50} of each material and its confidence limit at 95% were calculated by Litchfield - Wilcoxon method.

A. ACUTE TOXICITY WITH INTRAPERITONEAL ADMINISTRATION.

1. CAG

No death was observed up to 800mg/kg dose. Death occurred

at above 900mg/kg. The dose was increased by 100mg increment and at 1400mg/kg dose all animals were found dead. Unless animal died before the period of 24 hours, the rest were alive after 24 hours. In the case of death, the animal became immobile first at a few minutes after administration, then started writhing, clonic spasm, and developed hyperpnea, belly-crawling, and then dyspnea, ataxia and apnoea to result in the death. The heart movement did not cease for a few minutes even after apnoea occurred.

LD_{50} (Confidence Limit at 95%) calculated by L-W method is as followings;

16%	900	$S=1.169$, $N'=57$, $fLD_{50}=1.169^{0.367}=1.06$
50%	1050	$\therefore LD_{50}=1050$ (990--1100)mg/kg i.p.
84%	1250	

2. C P G

No death was observed up to 1000mg/kg dose. Death occurred at above 1100mg/kg dose and with 1700mg/kg all animals were found dead. The toxic conditions were observed similarly to those by CAG. LD_{50}(95% C.L.) calculated by L-W method is as followings;

16%	1050	$S=1.203$, $N'=78$, $fLD_{50}=1.203^{0.318}=1.06$
50%	1260	$\therefore LD_{50}=1260$ (1190--1340)mg/kg i.p.
84%	1520	

3. M A G

One mouse was dead at 700-800mg/kg dose. As the dose level was increased gradually, the number of death increased, and at 1400mg/kg dose all animals were found dead. The toxic effect of MAG was almost similar to that of the above two test materials.

LD_{50}(95% C.L.) calculated by L-W method is as followings;

16%	870	$S=1.228$, $N'=75$, $fLD_{50}=1.228^{0.320}=1.07$
50%	1070	$\therefore LD_{50}=1070$ (1000--1140)mg/kg i.p.
84%	1310	

D. ACUTE TOXICITY WITH ORAL ADMINISTRATION.

1. C A G

Death was observed not until 9000mg/kg, but it started to occur at above 10000mg/kg. All animals died at 17000mg/kg p.o. Though the toxic condition appeared later than the one by intraperitoneal administration, the condition was similar to its effect.

LD_{50} and C.L. 95% calculated by L-W method are as following;

16%	10500	$S=1.207$, $N'=72$, $fLD_{50}=1.207^{0.327}=1.06$
50%	12700	$\therefore LD_{50}=12700$ (12000--13400)mg/kg p.o.
84%	15300	

2. C P G

No animal died at lower dose than 10000mg/kg dose and all animal died at 16000mg/kg; death was observed at above 11000mg/kg dose on.

The conditions at which the toxic effect appeared were similar to those by i.p. administration, however, the appearance delayed and the action was weak.

LD_{50} and C.L. at 95% calculated by L-W method is as follows;

16%	9700	$S=1.261$, $N'=42$, $fLD_{50}=1.261^{0.417}=1.10$
50%	12400	$\therefore LD_{50}=12400$ (11280--13620)mg/kg p.o.
84%	15400	

3. M A G

With this test material it was not possible to calculate out LD_{50} because the test solution was so high in viscosity that the liquid could not be pushed through the stomach canula. When compared to the ratio of LD_{50} of p.o. over i.p. administrations of either CAG or CPG, LD_{50} of MAG by p.o. administration can be assumed to be about ten times higher than that by i.p. injection, or above 10000mg/kg.

TABLE

ACUTE TOXICITY OF MOUSE BY EACH TEST MATERIAL

ROUTE OF ADMINIST. / SAMPLE	LD_{50} mg/kg (95% C.L.) in Mice		p.o. / i.p.
	i. p.	p. o.	
DAG	1050 (990--1110)	12700 (12000--13400)	12.1
DPG	1260 (1190--1340)	12400 (11780--13030)	9.8
MAG	1070 (1000--1140)	Est. 12800 ?	Est. 12. ?

As shown in the Table listed above, all of the test material had the remarkably low acute toxicity. While DPG had the lower toxicity than DAG at the time of i.p. administration, at p. o. administration no significant difference was observed between the two materials.

Obviously potassium effect was not appeared. Gastrointestinal absorption of potassium salt could be assumed to be a little better than that of ammonium salt from the index of p.o. over i.p..

There was no significant difference observed between the toxicities of MAG and DAG by i.p. administration. MAG could not be administered p.o. due to high viscosity of its solution. However, taking it as about equal as to DAG, LD_{50} of MAG by p.o. administration could be estimated to be twelve times higher than that by i.p. administration, or 12 g/kg.

No matter which test materials are, their indices of p.o. over i.p. are in the vicinity of 10. This means in effect that the absorption of the materials from gastrointestinal tract seems to be very poor.

Therefore, from the toxicological viewpoint, the test materials may be considered to be practically non-toxic, particularly at the time of the oral administration. From the viewpoint

of pharmacology, however, since great quantity of the mater needed to get effect by oral administration, the use of the considered to be disadvantageous. Highly water soluble mat like DPG or DAG is rather recommended for medicinal use.

TOXICITY TEST OF

DI-POTASSIUM GLYCYRRHIZINATE
AND
DI-AMMONIUM GLYCYRRHIZINATE

MARUZEN PHARMACEUTICAL CO., LTD.

TOXICITY TEST OF
DI-POTASSIUM GLYCYRRHIZINATE
AND
DI-AMMONIUM GLYCYRRHIZINATE

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Professor K. Okamoto
Dept. of Pathology
School of Medicine
Kyoto University

TEST MATERIAL

(Products of Maruzen Pharmaceutical Company Limited)

Di-Potassium Glycyrrhizinate (D P G) $C_{42}H_{60}O_{16}K_2 \cdot 2H_2O = 935.14$

Di-Ammonium Glycyrrhizinate (D A G) $C_{42}H_{60}O_{16}(NH_4)_2 = 857.022$

I. ACUTE TOXICITY TEST
(Single Dose)

A group of six healthy mice weighing 15 to 17 g. regardless of the sex, was used for each materials being tested. The five to 10% aqueous solution of the material was administered either intraperitoneally or orally to the animals, which were kept until 7 days later and observed after 168 hours for the mortality. From the result obtained LD_{50} and confidence limit at 95% were calculated by Lictchfield-Wilcoxon method.

A. ACUTE TOXICITY BY INTRAPERITONEAL ADMINISTRATION.

1. D P G

The solution used was 10% aqueous. No death observed until 1000mg/kg dose. Death started to occur after 1100mg/kg and from that point on, the dose was increased by 100mg increment. Fifteen doses up to 2000mg/kg were observed. Those that died were found

dead within 24 hours. All those that survived were found to have survived during the 168 hour period. With the dose at which death was observed, the material caused animals to gradually increase their writhing, and clonic apnea after several minutes of resting after administration. They developed hyperpnea and started belly-crawling. They became dyspnea and ataxia, and then apnea to die within half an hour. The heart movement did not cease yet at the moment of death. Some showed stretching effect immediately after administration. Since KCl can cause stretching, it may be assumed to be caused by potassium.

LD_{50}(95% C.L.) after 7 days was calculated as followings by L-W method.

16%	1050
50%	1400
84%	1760

$S=1.3$, $N'=60$, $fLD_{50}=1.3^{0.357}=1.095$

$\therefore LD_{50}=8100$ (1280--1540)mg/kg i.p.

2. D A G

The solution used was 5% aqueous. No death was observed up to 800 mg/kg dose. The death started appearing at above 900mg/kg dose. From that on, the dose was increased by 100mg increment up to 2000mg/kg. Total of 12 doses was observed. In this case, too, those that died were found dead mostly within the period of 24 hours and those that survived survived until 7 days later. Toxic indications at the dose which gave death were more or less, similar to those by DPG, but stretching effect was not noted. This can be understood from the fact that ammonium chloride dose not cause stretching. The followings are LD_{50} (95%C.L.) after the period of 7 days calculated by L-W method.

16%	950
50%	1250
84%	1530

$S=1.27$, $N'=42$, $fLD_{50}=1.27^{0.427}=1.11$

$\therefore LD_{50}=1250$ (1130--1390)mg/kg. i.p.

B. ACUTE TOXICITY BY ORAL ADMINISTRATION.

1. D P G

With the use of stomach cannula, the 10% solution was administered orally. The dose was increased by 500mg increment from 4000mg/kg dose. For each group given 5000mg/kg and 6500 mg/kg doses, only one mouse of each group died but the rest survived. At above 7000 mg/kg dose animal started dying. Then the dose was increased to 10000mg/kg by twenty dose increment and the animals were observed until 7 days later. In this case, too, most of those that died was found dead within 24 hours and those that survived survived for 7 days.

The LD_{50} (95% C.L.) calculated by L-W method is as followings;

16%	6500	$S=1.24$, $N'=132$, $fLD_{50}=1.24^{0.241}=1.05$
50%	8100	$\therefore$ $LD_{50}=8100$ (7700--8500) mg/kg. p.o.
84%	9900	

2. D A G

Both 3% and 7% solution were used. No death was observed at less than 5000mg/kg dose, and the death started appearing at 5600 mg/kg. From that dose on, the dose was increased up to 11900mg/kg by 12 dose increment and observation was made. In this case, too, the death case was almost observed within 24 hours, and those that survived did survive for 7 day period.

LD_{50}(95% C.L.) calculated by L-W method is as followings;

16%	8200	$S=1.19$, $N'=42$, $fLD_{50}=1.19^{0.427}=1.077$
50%	9600	$\therefore$ $LD_{50}=9600$ (8800--10300)mg/kg. p.o.
84%	11600	

Both DPG and DAG have very low acute toxicty. Also their action is fairly acute and the judgement as to whether they cause death or survival is made almost within 24 hours. From the observation that those who survived could instantly recover from

the toxic action, they seemed to cause no cumulative nor residual effect. No marked effect was noted as far as the so-called potassium effect in the case of DPG.

In the intraperitoneal administration, not much significant difference in LD_{50} was found between DPG and DAG.

In the oral administration, on the other hand, DAG showed a little weaker result. But again the difference was tribial. Yet, looking at the ratio of p.o. over i.p., it was found that DPG was about 5.8 whereas DAG was 7.7. Although it would be too urgent to estimate the degree of gastrointestinal absorption immediately from the value of p.o. over i.p. ratio at lethal dose 50, if permissible to estimate, neither DPG nor DAG are absorbed well from gastrointestinal tract, but DAG is absorbed more poorly than DPG.

TABLE I. MOUTH TOXICITY OF DPG AND DAG ON MICE.

ROUTE OF ADMINIST. / DRUGS	LD_{50} mg/kg (95% C.L.) in Mice		P.O. / I.P.
	I. P.	P. O.	
D P G	1400 (1280 -- 1540)	8100 (7700 -- 8500)	5.79≒5.8
D A G	1250 (1130 -- 1390)	9600 (8800 -- 10300)	7.68≒7.7

II. SUBCHRONIC TOXICITY TEST

(Continuous Dose)

Ten groups of 5 healthy Wistar rats weighing 80 to 90 g. were raised in the fodder cage individually. The grouping was made by sex as well. To the fodder, Japan Blended Fodder KK CA-1 powder was used. The test material was incorporated in the fodder 0.1% in one group and 0.5% in the other. For the control only the above foddr was used.

Thus the groups of rats, each of which consisted of five rats, were divided in the following ten combinations;

	male	female
	control	control
fodder with DPG	0.1%	0.1%
	0.5%	0.5%
fodder with DAG	0.1%	0.1%
	0.5%	0.5%

The body weight of the rats and the amount intake of the fodder were measured daily. The amount intake of the fodder was measured by difference between a certain weight of the fodder measured the day before and the amount left out the day after.

After ninty days the animals were killed under ether anaesthetization with the blood taken out of the body. The internal organs were removed and particularly the weights of the liver, the kidney, the spleen, and the adrenals were measured. They were then submitted to make tissue sample by fixing them into 10% formalin solution.

I. BODY WEIGHT

As shown in Table II, the first control group of five rats gained their average weight by 280 g. in the male group and 127 g. in the female group during 90 day period.

On one hand the gaining of the body weight among those fed with the fodder containing 0.1% DFG was not so inferior to that among the control groups, showing the increases by 234 g. in the male rats and 114 g. in the female rats. On the other hand, among those fed with 0.5% DFG containing fodder, the female rat gained as much as the controls, showing the increase of the weight by 140 g., but the male rat gained rather inferiorly than compared to the control, showing the increase of the weight only by 179 g..

For those fed with 0.1% DAG in the fodder, the male rats gained 252 g. and the female 127 g., which were about the same as for the control. For those fed with 0.5% DAG, however, the weight gain by the female rats, 126 g., was again not much different from the control, but the one by the male rats, 218 g. showed the tendency of the lowering of gaining weight.

2. FODDER INTAKE

As summarized in the Table III, not much variation of the daily intake of the fodder was noticed among all groups of an animals being tested. Strictly speaking, though, when the time at which average intake of fodder lowered below 10% of the average weight was considered, the groups fed with DFG or DAG seemed to the little behind the control group. However, since there was not marked difference in the amount of intake by both groups fed with 0.1% and 0.5%, the reduction of appetite due to the presence of the drugs could not be conceivable. Rather, the difference in the amount of intake between male and female rats resulted in effect the increase of the absolutbe amount of intake of the drug by male rats fed with 0.5% drug; hence causing the depression of the increase in the body weight.

3. WEIGHTS OF THE REMOVED INTERNAL ORGANS.

As shown in the Table IV, among the internal organs which were removed at 90 days, after feeding, the weights of the liver, the

kidney, the spleen, and the adrenal glands were about the same difference among control groups and those fed with the drug.

The weight of the spleen of the male groups fed with the drug was less than compared to the control group, but the difference was not so great when its weight ratio against the body weight was taken into account. Although the effect especially on the adrenal gland of the groups fed with the drug was thought to be great, it turned out to be no great difference present.

As shown in the Table V, the tissue samples were prepared, and the microscopical observations were summarized in the Tables VI through VIII.

There were cases of pneumonia, appearance of small vacuole in the liver (probably of glycogen) observed in every groups of both sexes fed either 0.1% or 0.5% of either DPG or DAG. Also in the male groups fed with 0.5% DPG, there was pyelonephritis observed in one case. However, the former two conditions were also indicated in the control group as well as the group being tested. Also pyelogephritis is the condition that is often found in the white mice in general. Therefore they cannot be thought to be particularly due to the effect of the drugs being tested.

In summary, there was no pathological and histological toxicity recognized in the drugs being tested in this test.

CONCLUSION

As far as the results obtained from the test carried out for acute toxicity in mice and subchronic toxicity in rats which were given the continuous dose and kept for 90 days are concerned, both di-potassium glycyrrhizinate and di-ammonium glycyrrhizinate can be said to exhibit extremely lower toxicity.

TABLE II. AVERAGE BODY WEIGHT INCREASE.

(Average of five animals g ± S.E.)

TEST ANIMALS			WEIGHT in GRAMS after the DAYS of							Average body-weight increase
			0.	15.	30.	45.	60.	75.	90.	
CONTROL		m	86 ± 3	144 ± 4.8	204 ± 13.9	264 ± 13.9	303 ± 19	345 ± 20	366 ± 23.7	+ 280
		f	89 ± 3.4	125 ± 4.5	153 ± 3.2	187 ± 4.4	196 ± 5.0	213 ± 5.1	216 ± 5.6	+ 127
DPG	0.1%	m	85 ± 1.8	133 ± 3.3	179 ± 6.4	241 ± 7.8	270 ± 9.3	305 ± 11.4	319 ± 14.1	+ 23[illegible]
		f	89 ± 4.8	126 ± 7.3	147 ± 7.9	178 ± 6.3	188 ± 7.2	203 ± 6.5	203 ± 7.9	- [illegible]
	0.5%	m	85 ± 2.6	127 ± 7.5	161 ± 3.2	202 ± 7.3	244 ± 4.3	256 ± 8.4	264 ± 7.4	- 179
		f	88 ± 3.7	133 ± 6.1	157 ± 5.3	188 ± 6.3	201 ± 7.6	215 ± 4.6	228 ± 8.3	- 140
DAG	0.1%	m	85 ± 4.4	136 ± 6.3	186 ± 10.4	238 ± 16.5	282 ± 15.2	312 ± 17.2	337 ± 16.0	+ 252
		f	89 ± 4.1	129 ± 5.6	151 ± 7.1	182 ± 8.9	195 ± 10.9	209 ± 13.4	216 ± 16.2	+ 127
	0.5%	m	87 ± 1.6	124 ± 3.6	170 ± 4.2	231 ± 8.7	256 ± 12.7	278 ± 12.7	305 ± 14.7	- 218
		f	88 ± 4.8	129 ± 8. 8	156 ± 7.9	187 ± 7.3	197 ± 5.9	209 ± 4.0	214 ± 4.7	+ 126

TABLE III. AVERAGE AMOUNT of FODDER INTAKE (g.)

TEST ANIMAL		SEX	WEIGHT in GRAMS after the DAYS of 0.	15.	30.	45.	60.	75.	90.
CONTROL		m	13.3 (86)	20.8 (144)	19.8 (201)	22.9 (263)	24.6 (303)	19.9 (345)	17.5 (366)
		f	10.7 (89)	16.8 (125)	14.1 (153)	16.5 (187)	14.3 (196)	16.4 (213)	10.4 (216)
DFG	0.1%	m	11.0 (85)	18.2 (127)	17.5 (161)	18.0 (202)	19.3 (245)	13.6 (258)	13.0 (264)
		f	10.2 (89)	16.7 (126)	15.9 (147)	14.9 (178)	15.2 (188)	15.1 (203)	13.1 (203)
	0.5%	m	9.2 (85)	20.3 (133)	18.2 (180)	22.7 (241)	18.8 (270)	18.5 (305)	17.3 (319)
		f	8.6 (88)	20.5 (133)	18.1 (157)	16.5 (188)	15.8 (201)	15.4 (215)	17.1 (228)
DAG	0.1%	m	12.3 (85)	18.2 (136)	20.1 (186)	18.2 (238)	23.2 (282)	19.0 (311)	21.7 (337)
		f	10.3 (89)	16.4 (129)	15.3 (151)	21.9 (182)	20.7 (195)	16.4 (209)	16.8 (216)
	0.5%	m	9.6 (87)	16.7 (124)	19.8 (170)	21.9 (231)	20.1 (256)	21.6 (278)	19.6 (305)
		f	11.6 (88)	18.4 (123)	17.5 (156)	17.8 (187)	16.4 (197)	15.8 (209)	13.6 (214)

Note: the numbers in the brackets ( ) indicates the average body weight.
Those that are underlined are the days when the fodder consumption went below 10% of the body weight. They do not correlate each other in the strict sense because of the 15 day intervals.

TABLE IV. WEIGHT OF INTERNAL ORGAN TAKEN OUT at 90 DAYS LATER.

(Average weight and standard deviation of 5 animals of each group.)

TEST ANIMALS			LIVER (g.)	KIDNEY (g.)	SPLEEN (g.)	ADRENALS (mg.)
CONTROL		m	12.5±0.62 (3.5)	2.9±0.14 (0.80)	0.9 ± 0.2 (0.25)	44 ± 3.7 (0.012)
		f	8.3±0.60 (3.8)	1.9±0.02 (0.87)	0.6±0.08 (0.27)	52 ± 3.3 (0.024)
DFG	0.1%	m	10.1±0.58 (3.8)	2.5±0.05 (0.94)	0.6±0.05 (0.22)	41 ± 2.3 (0.013)
		f	8.9±0.41 (4.4)	1.8±0.08 (0.88)	0.5± 0.01 (0.24)	53 ± 2.4 (0.026)
	0.5%	m	12.0±0.60 (3.8)	2.9±0.18 (0.90)	0.62±0.04 (0.19)	42 ± 25 (0.016)
		f	9.3±0.40 (4.1)	2.2±0.24 (0.96)	0.52±0.05 (0.22)	52 ± 2.9 (0.023)
DAG	0.1%	m	11.5±0.14 (3.4)	2.5±0.18 (0.74)	0.66±0.05 (0.20)	43 ± 2.1 (0.013)
		f	7.5±0.50 (3.5)	1.7±0.14 (0.78)	0.50±0.05 (0.23)	56 ± 4.0 (0.025)
	0.5%	m	12.8±1.29 (4.2)	2.6±0.18 (0.85)	0.62±0.05 (0.20)	40 ± 3.3 (0.013)
		f	7.3±0.24 (3.4)	1.8±0.12 (0.84)	0.54±0.02 (0.20)	52 ± 2.9 (0.024)

Note: The brackets ( ) in the table indicates (weight of internal organ) / (body weight) X 100.

TABLE V. TISSUE MARK and NUMBER of TISSUE SAMPLE of TEST ANIMALS. *

of ANIMALS	CONTROL										DPG 0.1%										DPG 0.5%				
	M					F					M					F					M				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
TISSUE MARK	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}	K_1	K_2	K_3	K_4	K_5	K_6	K_7	K_8	K_9	K_{10}	K_{11}	K_{12}	K_{13}	K_{14}	K_{15}
NUMBER OF TISSUE SAMPLE	10	10	10	10	10	8	9	9	10	11	9	9	9	8	9	8	9	8	9	9	8	8	8	8	10
ORGANS THAT ARE MADE INTO TISSUE SAMPLES: BRAIN																									
HEART																									
LUNG																									
LIVER																									
SPLEEN																		—							
STOMACH																									
PANCREAS	—	—	—	—	—	—	—	—			—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
THYMUS						—										—									
KIDNEY																									
ADRENALS											—	—	—	—	—		—				—	—	—	—	
GONADS						—	—	—	—					—		—		—	—	—	—	—	—	—	

TEST ANIMALS	DPG 0.5%					DAG 0.1%										DAG 0.5%									
	F					M					F					M					F				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
TISSUE MARK	K_{16}	K_{17}	K_{18}	K_{19}	K_{20}	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_{10}	A_{11}	A_{21}	A_{31}	A_{41}	A_{51}	A_{61}	A_{71}	A_{81}	A_{91}	A_{101}
NUMBER OF TISSUE SAMPLE	8	8	8	8	8	8	11	11	11	11	11	11	11	11	11	11	11	11	11	11	10	10	9	11	10
ORGANS THAT ARE MADE INTO TISSUE SAMPLES: BRAIN																									
HEART																						—			
LUNG																									
LIVER																									
SPLEEN																									
STOMACH						—																	—		
PANCREAS	—	—	—	—	—																				—
THYMUS						—																	—		
KIDNEY																									
ADRENALS	—	—	—	—	—																				
GONADS	—	—	—	—	—																				

— Indicates organs not taken out.

TABLE VI. TISSUE OBSERVATIONS of ALL ORGANS of the TEST ANIMALS (3).

TEST ANIMALS	CONTROL									
	MALE					FEMALE				
	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}
tissue mark	1	2	3	4	5	6	7	8	9	10
BRAIN	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
HEART	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
LIVER	NB	NB	SVHC	NB	SVHC	NB	NB	HCG	NB	NB
LUNG	NB	PI	NB	NB	I	CB	NB	IH&C	I	IHCM
SPLEEN	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
STOMACH	NB	NB	NB	NB	NB	NB	NB	BN	NB	NB
PANCREAS	-	-	-	-	-	-	-	-	NB	NB
INTESTINE	NB	NB	NB	NB	NB	NB	-	NB	NB	NB
ADRENALS	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
GONADS	NB	NB	NB	NB	NB	NB	-	-	-	-
KIDNEY	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB

Note: - : Organs not taken out.
NB : No significant change.
SHVC : Small Vacuoles in HepaticCell.
HCC : Hepatocellular Granulation.
PI : Partial Interalveolitis.
I : Interalveolitis.
IH&C : Interalveolar Hypertrophy & Congestion.
CB : Chronic Bronchitis
IHCM : Interalveolar hypertrophy & Congestion & Mild.

TABLE VII. TISSUE OBSERVATIONS of ALL ORGANS of the TEST ANIMALS (2).

TEST ANIMALS	D					P					G									
	0.1% male					0.1% female					0.5% male					0.5% female				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
TISSUE MARK	K_1	K_2	K_3	K_4	K_5	K_6	K_7	K_8	K_9	K_{10}	K_{11}	K_{12}	K_{13}	K_{14}	K_{15}	K_{16}	K_{17}	K_{18}	K_{19}	K_{20}
BRAIN	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
HEART	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
LIVER.	NB	NB	PHG	PHG	NB	PVHC	NB	NB	NB	NB	NB	NB	NB	NB	PVHCE	HCG	NB	HCG	NB	SVHC
LUNG	nb	CON	SI	PA	SI	CB	NB	BCI	NB	CB	NB	IBR	NB	IBR	NB	I	C,PI	NB	NB (PI)	SI
SPLEEN	NB	NB	NB	NB	NB	NB	NB	—	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
STOMACH	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
PANCREAS	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
INTESTINE	NB	NB	NB	NB	NB	—	NB	BI	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
ADRENALS	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	PN	NB	NB	NB	NB	NB	NB	NB	NB	NB
KIDNEY	—	—	—	—	—	NB	—	NB	NB	NB	—	—	—	—	NB	—	—	—	—	—
GONADS	NB	NB	NB	—	NB	—	NB	—	—	—	—	—	—	—	NB	—	—	—	—	—

Note: CON : Congestion. SI : Slight Interalveolitis. PA : Pulmonary Abscess. BCT : Bronchitis.
IBR : Inflammation at Bronchial Region. C,PI : Congestion, Partial Interalveolitis.
PHG : Partial Hepatocellular granulations. PVHC : Partial Vacuoles in Hepatic Cells.
" E: " hepatic cell edge .

TABLE VIII. TISSUE OBSERVATIONS of ALL ORGANS of the TEST ANIMALS (3).

TEST ANIMALS	D										A					G				
	0.1% male					0.1% female					0.5% male					0.5% female				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
tissue mark	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_{10}	A_{11}	A_{12}	A_{13}	A_{14}	A_{15}	A_{16}	A_{17}	A_{18}	A_{19}	A_{20}
BRAIN	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
HEART	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
LIVER	NB	NB	NB	NB	IHSV	IHSVn	IHSVn	IHSVn	IHSVn	NB	IHSV	IHSV	NB	NB	NB	IHV	NB	IHSV	NB	NB
LUNG	SI	SI	PIH	I	C,PH	LI	NB	IBR	IBR	PIH	NB	PI	NB	NB	NB	NB	BV	IBR	CD	BOT
SPLEEN	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
STOMACH	—	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	—	NB	NB
PANCREAS	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	—
INTESTINE	—	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	—	NB	NB
ADRENALS	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	B	B NB	NB	NB	NB	NB	NB	NB
KIDNEY	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
GONADS	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB

Note: PIH : Partial interalveolitis hypertrophy.
LI : Limited Interalveolitis.
IHV : IntraHepatocellular Vacuoles.
C,PH : Congestion, Partial Hemorrhage.
IHSV : Intrahepatocellular small vacuole.
" n: " numerous " "

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THE EFFECTS OF GLYCYRRHETINIC ACID ON SALT AND WATER METABOLISM

BY

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Revers (1948) observed that succus liquiritiae caused water retention in some peptic ulcer patients. Molhuysen, Gerbrandy, de Vries, de Jong, Lenstra, Turner, and Borst (1950) confirmed this and found an extract consisting mainly of glycyrrhizin to be equally effective.

In normal persons, the authors reported that the succus made the potassium balance markedly negative and the sodium balance strongly positive. They failed to produce similar results in a patient with Addison's disease and concluded that the drug acted through the adrenals.

Groen, Willebrands, and Kamminga (1951), and Groen, Frenkel, Kamminga, and Willebrands (1952) found liquorice to have a DOCA-like effect on sodium and potassium metabolism in normal people and in Addisonian patients. They found glycyrrhizinic acid to be the active principle and used it for the treatment of Addison's disease.

Borst, ten Holt, de Vries, and Molhuysen (1953) suggested that liquorice activity on salt and water metabolism is potentiated by the endogenous corticoids. No conclusive evidence of such a potentiation has been found in the treatment of rheumatoid arthritis (Hart and Leonard, 1954) or in the increase in the urinary output of 17-ketosteroids (Hudson, Mittelman, and Mann, 1953).

Calvert (1954) successfully maintained a patient with Addison's disease on liquorice extract for over a year. The daily maintenance dose decreased from 60 g. to 3 g. The author concluded that the drug has a place in long-term therapy of this disease.

Glycyrrhizinic acid is a conjugate of glycuronic acid and glycyrrhetinic acid ; the latter was found to have a DOCA-like effect in normal people and in Addisonian patients (Card, Mitchell, Strong, Taylor, Tompsett, and Wilson, 1953).

Nelemans and Stamperius (1949) and Card *et al.* (1953), however, found no evidence of any effect of liquorice or glycyrrhetinic acid on water and electrolyte metabolism in laboratory animals.

Glycyrrhetinic acid is not a steroid and therefore it would be of great interest if it could be shown to possess some of the more important properties of the corticosteroids.

METHODS

Urinary Sodium Excretion

The details of the method used for assaying mineralo-cortical activity will be reported separately. Young female albino rats, adrenalectomized under ether anaesthesia three days before the assay, were used. The rats were divided into groups of 5–7 animals each. On the day of the experiment, each rat was given two loads of the electrolytes investigated. Each group received either a known quantity of glycyrrhetinic acid dissolved in propylene glycol or a known quantity of DOCA in the same volume of the solvent. A comparable group of animals was included in every experiment and received the same quantity of solvent alone.

The urine of each animal was collected separately over a period of six hours and its sodium or potassium content was estimated by the flame-photometer. In some experiments, the urine was collected for 4 hr. only.

Blood Electrolytes

Male albino rats weighing 300 g. were adrenalectomized under ether anaesthesia and kept on rat cubes and a drinking solution containing sodium chloride (1%) and glucose (5%) for 4 days. On the 5th day, the animals were weighed and divided into three groups of equal mean weight. Injections were started and continued daily for five days. No saline drink was administered during this period.

Group I rats received subcutaneous injections of 0.25 ml. propylene glycol. Group II received 3 mg. glycyrrhetinic acid dissolved in 0.25 ml. propylene glycol. Group III received 1 mg. of DOCA in 0.25 ml. propylene glycol. Group IV rats were non-adrenalectomized, from the same colony, and of the same weight. Each rat received 0.25 ml. of propylene glycol subcutaneously.

On the 5th day, 4 hr. after the injection, the animals were anaesthetized with an intraperitoneal injection of 0.7 ml. 6% pentobarbitone sodium solution. The chest was opened and the heart punctured. The blood was collected in a centrifuge tube and allowed [illegible]

* In receipt of a scholarship from Cairo University.

the serum was pipetted off later. After dilution, the sodium and potassium contents were estimated with the flame photometer.

Diuresis

(*a*) *Anaesthetized Rats.*—Male rats above 250 g. were used. They were fasted overnight, but given free access to tap water. At about 10 a.m. anaesthesia was produced by administering 5 ml./100 g. body weight of 10% alcohol in tap water into the stomach by a thin rubber catheter, and the rat was kept in a warm place. If the rat was not unconscious in 45 min., another dose was given (1.5 ml. of the same solution/100 g. body weight). A small suprapubic cut was made, the bladder secured and a thin polythene tube (3 mm. internal diameter) was introduced into the bladder through the apex and tied in securely. The penis was tied to prevent any urine escaping except through the polythene tube. A thin polythene cannula was put into the jugular vein. A fine thermometer was introduced into the rectum and the animals were placed on a box warmed by electric bulbs and their temperature kept as constant as possible at 37°. The end of the catheter was fixed to a graduated centrifuge tube and a reading of the urine collected was taken every 10 min. When diuresis started, a new water load of 10–14 ml. of water, or of 3% alcohol in tap water if anaesthesia was getting light, was introduced by stomach tube. The degree of anaesthesia was kept deep enough to prevent the animals from moving or responding to external stimuli, but not so deep as to inhibit diuresis. Whenever 8 ml. of urine had been collected, it was replaced by 10 ml. of water by stomach tube or of 3% alcohol, if required. The solvent of the drug was administered intravenously in the same manner as the drug, which was given as a clear solution in propylene glycol (0.05–0.2 ml.) and washed down the cannula with a minimum amount of saline. Care was taken, as all these factors affected the flow of urine.

(*b*) *Conscious Rats.*—Female albino rats around 150 g. weight were used. Food was withdrawn at 5 p.m. the previous night; water was withdrawn at 9 a.m. on the day of the experiment. The animals were divided into two equal groups, one to act as control and the other as test. At 11 a.m. each control rat was given 1 ml. of propylene glycol by stomach tube. The test group had 100 mg. glycyrrhetinic acid dissolved in 1 ml. of propylene glycol. The rats were kept in a quiet place for 3 hr. At 2 p.m. each animal got 12 ml. of tap water by stomach tube; its bladder was emptied by a gentle pulling of the tail, and the rat was placed in a metabolism cage which consisted of a waxed tin funnel covered by a waxed wire mesh and surmounted by a cylinder of wire mesh with a tin cover. A graduated cylinder was placed under each cage to receive the urine. Two hours after placing the animals in the cages the bladder was emptied by pulling the tail gently, and the urine was allowed to run into the cylinder; then a reading was taken. The whole experiment was carried out at constant temperature (22° C. ± 1°) in a quiet room. Five days later, after 4 days of complete rest, the experiment was repeated and the groups were crossed so that each animal acted as its own control. To eliminate any source of emotional stress that may interfere with the diuresis, it was necessary first to train the animals to the conditions of the experiment, particularly the use of the stomach tube, for a week.

(*c*) *Normal Dog.*—A collie bitch of 10 kg. weight was used. A period of 3 weeks' training was necessary before the full co-operation of the dog was gained.

The night before the experiment, food was removed from the cage; the dog had free access to water. At 9 a.m. the next morning, 70 ml. of tap water, divided into three portions, was given by stomach tube. Glycyrrhetinic acid (350 mg.) was suspended in the first portion and washed down by the last two. The dog was then allowed free movement with no access to food or water for 2 hr. At 11 a.m. a self-retaining catheter was introduced and the bladder emptied. The dog was placed in a Pavlov stand. At 11.15 a.m. 250 ml. of tap water was given by stomach tube, followed by 300 ml. at 11.55. From 12 noon, urine flow was recorded at 5 min. intervals for 1 hr. and 40 min. The total urine excreted from 11.15 till 3.05 was also measured. Throughout the experiment, everything was avoided which might antagonize the dog and all external interferences were cut down as far as possible. A constant temperature and complete quietness were essential.

In one experiment in which the dog received no glycyrrhetinic acid, 2.5 mg. DOCA was injected subcutaneously at 9 a.m. and the rest of the experiment continued as before.

(*d*) *Rats with Diabetes Insipidus.*—Female albino rats (120–150 g.) were used. The posterior lobe of the pituitary gland was sucked out under ether anaesthesia, most of the anterior lobe being left intact. The operation was kindly performed by Dr. M. Vogt. The rats were given 5% glucose drinking solution for 2 days and ordinary rat cubes for food. Penicillin was administered as a protective measure for 2 days. In order to discover which rats were diabetic, 2 days after the operation each rat was put in a metabolism cage and was freely supplied with water and cubes. Urine was collected over 24 hr. and the volume compared with that from intact rats of the same batch. Later, collection of urine was carried out over-night for 18 hr. with water only supplied, the feeding being done late every afternoon. The urine volume was again compared with that of intact control rats. Overnight urine collections were continued during the glycyrrhetinic acid tests to measure the degree of diabetes insipidus present in every test rat during the whole procedure. This was necessary because in some of the operated rats only a transient phase of diabetes was achieved.

The test for the antidiuretic action of glycyrrhetinic acid was carried out only on those rats in which the operation proved successful. All the rats used in these tests were diabetic throughout the test period.

The method used to test the effect of glycyrrhetinic acid on diuresis in the rats with diabetes insipidus was the same as that employed for the non-anaesthetized intact rats. The same dose (100 mg.) of the acid was introduced by stomach tube 3 hr. before the 12 ml. water load. A 2 hr. urine collection was carried out. Each rat acted alternately as control and as test animal. Two control values and two test values were obtained from each rat whenever possible. Before subjecting the rats to the operation they were trained for one week to get them accustomed to the stomach tube and urine collection procedures.

Water Absorption from the Alimentary Canal

Normal Rats.—Adult albino rats of about 170 g. weight were trained for one week to being given water by stomach tube. The actual experiment consisted in fasting the rats overnight, allowing them free access to drinking water. They were then divided into pairs, selected to be identical in sex and weight. At 11 a.m. 1 ml. of propylene glycol was administered by stomach tube to one rat of each pair and 100 mg. of the acid suspended in the same volume of propylene glycol to the other. The rats were kept in a quiet, warm place for 3 hr., when they received 5% of the body weight of warm tap water by stomach tube. They were killed 15 to 60 min. later and their intestinal tracts were dissected out and weighed after removing the formed contents of the rectum and caecum.

Throughout the experiment, the two rats of each pair were handled together and kept in the same cage; conditions for the members of the same pair were therefore as identical as possible and the results obtained for the control and the glycyrrhetinic-treated rats comparable.

The dose of the acid, its mode of administration, and the period elapsing until the administration of the water load were chosen so as to duplicate the procedures in the diuresis experiments reported before, in the hope that such an arrangement would facilitate the assessment of the results.

In another series of experiments, a 300-mg. dose of the acid was given to the test animals, the contents of the caecum were not emptied before weighing the gut, and the liver was dissected out and weighed. The times allowed for water absorption were 15, 30, and 75 min.

RESULTS

Urinary Sodium Excretion

In glycyrrhetinic-acid-treated adrenalectomized rats (Table I) sodium excretion was not inhibited or altered to any significant degree if the collection of urine was continued for a period sufficiently long to allow urine to flow. With doses of 0.2 and 0.4 mg. glycyrrhetinic acid a 6 hr. collection was sufficient. When DOCA, 1.0 μg./rat, was administered under the same conditions significant sodium retention was achieved ($P=0.03$).

TABLE I

SODIUM EXCRETION IN ADRENALECTOMIZED RATS

Expt. No.	Time of Collecting Urine (hr.)	Propylene Glycol Only		Glycyrrhetinic Acid (GA) or DOCA			
		No. of Rats	Mean Na Excretion (mg.)	No. of Rats	Dose (μg.)	Mean Na Excretion (mg.)	Na Excretion (% of Control)
1	6	7	3·45	8	400 GA	3·78	109·6%
2	6	6	0·61	6	200 GA	1·45	240·0%
3	6	7	2·66	7	200 GA	2·25	84·6%
				7	1·0 DOCA	0·899	29·6%
4	6	5	2·53	6	400 GA	2·23	89·2%
				5	5·0 DOCA	0·573	22·6%
5	6	6	1·58	6	400 GA	1·29	84·3%
6	4	7	1·48	4 and 3*	400 GA	0·56	37·8%
7	4	7	1·66	4 and 3*	400 GA	0·39	23·5%
				7	1·5 DOCA	0·43	26·0%

* Number of animals which excreted no urine at all. They are included in the calculation of the mean Na excretion for the whole group.

When urine was collected over 4 hr. only, and a dose of 0.4 mg. of the acid was administered to each rat, the mean sodium excretion for the whole group was appreciably below that of the control group. Under these conditions, however, it was observed that a number of the glycyrrhetinic-acid-treated rats did not excrete any urine at all and thus did not contribute to the total excretion of sodium by the group. With a 4 hr. collection, 1.5 μg. DOCA caused a significant sodium retention ($P<0.01$), while the volume of urine collected from any rat was not appreciably smaller than that collected in 6 hr. In other experiments, the second sodium load was replaced by a load of 3.8 mg. potassium chloride in 2.0 ml. water. The group of rats receiving 400 μg. of glycyrrhetinic acid showed a significant increase in their mean urinary potassium output compared with the control group ($P<0.02$). See Table II.

TABLE II

POTASSIUM EXCRETION IN ADRENALECTOMIZED RATS AFTER A KCl LOAD OF 3·8 MG.

Controls				Glycyrrhetinic Acid (400 μg.)			
No. of Rats	Mean Na Excretion (mg.)	Mean K Excretion (mg.)	$\frac{K}{Na} \times 100$	No. of Rats	Mean Na Excretion (mg.)	Mean K Excretion (mg.)	$\frac{K}{Na} \times 100$
6	1·05	0·86	81·9	6	1·62	2·41	148·8

Blood Electrolytes

The results are shown in Table III. DOCA in 1.0 mg. doses given for five days successfully maintained the serum sodium of adrenalectomized rats above the normal level for the non-adrenalectomized rats of the same colony ($P=0.01$). The

TABLE III

EFECTS OF GLYCYRRHETINIC ACID (GA) ON SERUM ELECTROLYTES IN RATS

	Group	No. of Rats' Group	Mean Serum Na (% of Normal)	Significance of Na Change	Mean Serum K (% of Normal)	Significance of K Change	Mean Gain in Weight (g.)	Survival %
Normal	0·25 ml. propylene glycol	8	100	—	100	—	+0·1	100
Adrenalectomized	0·25 ml. propylene glycol	10	88·7	From normal P<0·001	166·7	From normal P=0·01 0·02	−10·5	30
	1 mg. DOCA	8	105·9	From adrenalectomized non-treated P<0·001 From normal P 0·01	144·7	From adrenalectomized, non-treated—not significant From normal—not significant (P 0·1 0·2)	+0·5	100
	3 mg. GA	10	91·3	From adrenalectomized non-treated—not significant From normal P<0·01	163·5	From adrenalectomized non-treated—not significant From normal P=0·2	−8·6	50

The doses are per day per rat of approx. 300 g. All injections were given subcutaneously for 5 days.

serum sodium of the DOCA-treated adrenalectomized rats was thus very much higher than that of the adrenalectomized propylene-glycol-injected controls ($P<0.001$). These controls showed the classical picture of adrenal deficiency; their serum sodium was below the normal level ($P<0.001$) and their serum potassium higher ($P<0.001$). The serum potassium of the DOCA-treated group showed a slight return towards normal, but was not significantly different from that of untreated adrenalectomized or normal rats.

Glycyrrhetinic acid did not cause a significant rise of serum sodium or a fall of serum potassium. The daily dose administered was equivalent to 600 mg. glycyrrhetinic acid for a 60 kg. man, a dose well within the range reported to be effective in man.

DOCA successfully maintained all the animals in the group alive and in good shape. The glycyrrhetinic acid group started to show weakness much earlier even than the adrenalectomized controls. The survival of the group, however, was slightly, but not significantly, longer than that of the controls.

The adrenalectomized animals treated with glycyrrhetinic acid or left without treatment showed a marked loss of weight; this was prevented by DOCA.

Diuresis

(*a*) *Anaesthetized Rats.*—Glycyrrhetinic acid caused an appreciable inhibition of diuresis. In a dose of 1 mg. intravenously to a 300 g. rat, it caused almost complete suppression of urine flow from which recovery was very slow, taking about 3–4 hr. The response, however, was not uniform. Some rats responded to a small dose of 200 μg. more markedly than other rats to bigger doses.

When given intravenously (Fig. 1) 0.1 ml. propylene glycol alone had no effect, whereas, when the same fluid contained 100 μg. of the acid, the diuresis diminished appreciably for 4 hr. and then did not regain the initial level, although this had previously been maintained practically constant for 2 hr.

The smallest effective dose for a 280 g. rat was around 40 μg. (Fig. 2); 150 μg. had a marked effect on this rat.

When the drug was injected intravenously, a slight haemorrhagic tinge was sometimes noticed in the urine. This tinge, however, was also noticed with propylene glycol alone.

(*b*) *Conscious Rats.*—The results are shown in Table IV. A variable degree of water retention

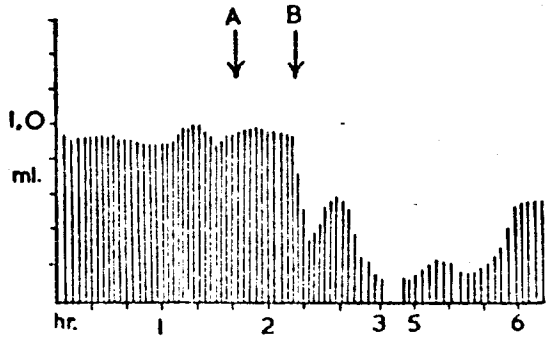

FIG. 1.—Effect of intravenous glycyrrhetinic acid on diuresis in a rat. Ordinate: Urine volume in ml. 10 min. Abscissa: Time in hr. A: 0·1 ml. propylene glycol washed in with [illegible] ml. [illegible] B: Same as A, but containing 100 μg. glycyrrhetinic acid

TABLE IV

EFFECT OF GLYCYRRHETINIC ACID (GA) BY MOUTH ON DIURESIS IN NORMAL UNANAESTHETIZED RATS

Urine collected over 2 hr. after a load of 12 ml. H_2O.

No. of rat	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Mean
Controls (ml. urine)	8	10	10	7·5	8	6·5	8·5	9	7·5	8·5	9·5	6	9	8	8·29
100 mg. GA (ml. urine)	3·5	4·75	3·5	6·5	6·5	6·5	5	1·5	7	4·75	7·25	4	7·25	5·5	5·25
$\frac{GA}{Control} \times 100$	44	48	35	87	81	100	59	17	94	56	76	67	81	69	65

was observed after the administration of 100 mg. of glycyrrhetinic acid, each rat being used as its own control.

The mean water retention of the whole group was 35% ; only one out of 14 rats showed no inhibition of diuresis.

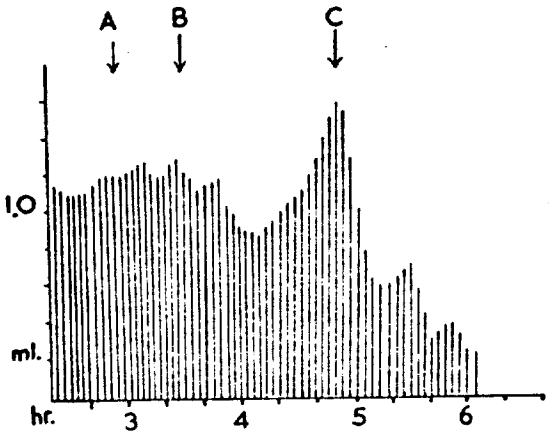

FIG. 2.—Effect of intravenous glycyrrhetinic acid on diuresis in a rat. Ordinate: Urine volume in ml. 10 min. Abscissa: Time in hr. A: 0·05 ml. propylene glycol washed in with 0·1 ml. saline. B: same as A but containing 40 μg. glycyrrhetinic acid. C: Same as A but containing 150 μg. glycyrrhetinic acid.

(*c*) *Normal Dog.*—In control experiments without glycyrrhetinic acid, a mean rate of diuresis of 4 ml./min. was observed (Fig. 3). Glycyrrhetinic acid caused a depression of the rate of diuresis averaging 40% during the peak period. The volume of urine excreted over 3 hr. and 50 min. dropped by an average of 20% in the glycyrrhetinic acid experiments. In the one experiment with DOCA no such inhibition was observed.

To see whether the antidiuretic effect of glycyrrhetinic acid was due to a lowering of the blood pressure, the femoral vein of a 200 g. rat under urethane anaesthesia was cannulated and connected to a saline-filled burette; the carotid artery was also cannulated and connected to a sensitive blood-pressure manometer. Propylene glycol produced a short-lived rise in blood pressure, and ethanol in the same dilution produced a smaller pressor effect. Glycyrrhetinic acid (0.4 mg. in 20% alcohol) certainly had no hypotensive effect; it caused a rise in pressure similar to that due to the solvent.

Since the antidiuretic effect of glycyrrhetinic acid could not be attributed to a fall in blood pressure, attempts were made to discover what mechanisms were involved. Rats in which removal of the posterior lobe of the pituitary had been attempted were used, and their responses compared with those of conscious normal rats (Table V). In order to be able to do cross-over tests, the diuresis was followed in the unanaesthetized animal and the drug given by mouth.

TABLE V

DIURESIS IN RATS AFTER REMOVAL OF THE POSTERIOR LOBE OF THE PITUITARY

The rats had free access to water; urine collected over 18 hr.

Rat Number	Urine Secretion as % of Control				
	6th Day after Operation	1st Experiment*		2nd Experiment*	
		Before	After	Before	After
1	190	93	311	—	—
2	141	224	239	376	372
3	190	93	710	—	—
4	270	195	533	106	286
5	1,400	1,830	350	370	330
6	2,360	3,170	510	650	400

* Before and after the corresponding glycyrrhetinic acid experiments (Table VI).

(*d*) *Rats with Diabetes Insipidus.* In Table V the degree of diabetes insipidus attained by the operated rats is presented. The values are calculated as percentage of urine secretion by non-operated controls of the same batch. The first values given are those for the sixth night after operation; they are followed by the figures for the night preceding, and that following, each glycyrrhetinic acid test. The figures in this table show that the degree of diabetes insipidus resulting from the operation was not steady from day to day. On the other hand, all rats showed a manifest increase in diuresis maintained till the animals died or were killed several weeks after the operation.

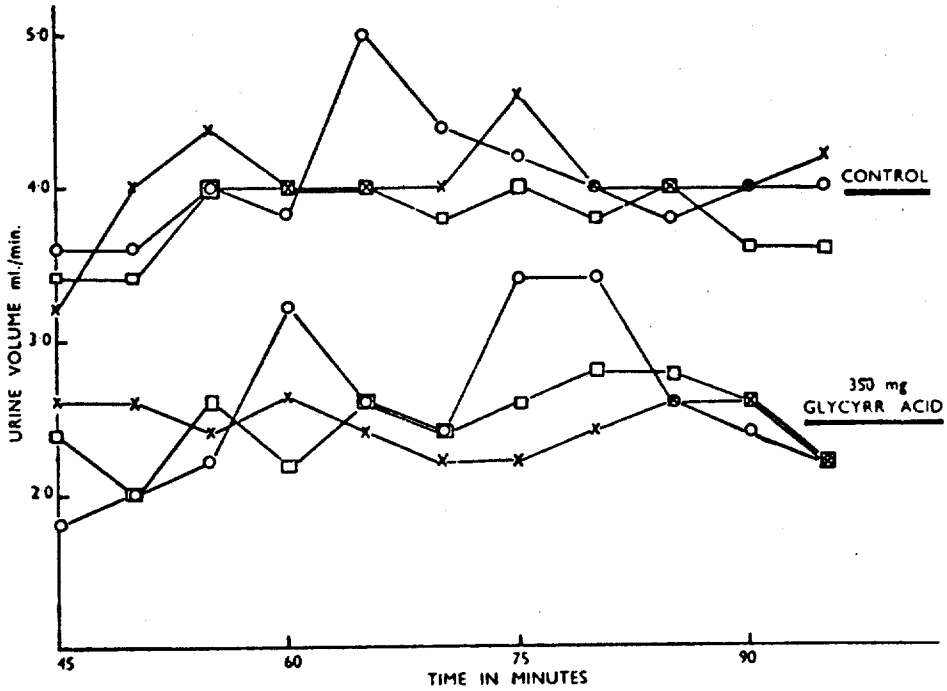

FIG. 3.—Five min. urine collections after two water loads of 250 ml. and 300 ml. Three tests and three control experiments carried out on the same dog. Ordinate: Urine volume in ml./min. Abscissa: Time in min.

When glycyrrhetinic acid was administered to these rats, an inhibition of diuresis was observed (Table VI). This inhibition was comparable to that met with in non-diabetic rats. The mean water retention in these experiments was 41.5% compared with 35% in the non-diabetic rats.

To see whether delayed water absorption from the gastro-intestinal tract played any part in the reduction of water excretion, the method used by Heller and Smirk (1932) was employed to investigate the effect of glycyrrhetinic acid on water absorption.

Water Absorption from the Alimentary Canal

In the first series of experiments (Table VII) the weight of the gut, when expressed as percentage of body weight, was always higher after glycyrrhetinic acid than in the control rats. This was so whether the rats were killed 15 or 60 min. after the water load. No significant difference in the degree of water retention was observed between the two groups. Glycyrrhetinic acid can be detected in the stomach as a white precipitate 4½ hr. after administration.

In the second series of experiments, the dose of glycyrrhetinic acid was trebled and three groups,

TABLE VI

EFFECT OF ORAL GLYCYRRHETINIC ACID ON DIURESIS IN RATS WITH DIABETES INSIPIDUS

Urine collected over 2 hr. after a load of 12 ml. H_2O.

No. of rat	1	2		3	4		5		6		Mean
No. of experiment		A	B		A	B	A	B	A	B	
Control day (ml. urine)	8·75	8·5	7·0	10·5	3·0	2·75	10·75	9·5	10·0	6·75	7·75
After 100 mg. glycyrrhetinic acid (ml. urine)	7·75	6·5	6·25	6·0	1·5	0·75	5·5	6·0	5·5	2·0	4·78
Test day / Control day × 100	88·5	76·5	89·0	57·0	50·0	27·0	51·0	61·0	[illegible]	30·0	[illegible]

TABLE VII

EFFECT OF ORAL GLYCYRRHETINIC ACID (GA) ON ABSORPTION OF WATER FROM THE GUT OF RATS

No. of Pair	Time After Giving Load (min.)	Wt. of Gut as % of Body Wt.		$\frac{b}{a} \times 100$*
		Control (a)	GA (b) 100 mg.	
1	15	8·0	8·9	111
2	15	8·6	9·6	112
3	15	9·0	9·4	105
4	15	7·4	8·6	116
5	15	8·3	10·0	120
	Mean	8·26	9·3	113
1	60	6·2	7·2	117
2	60	6·6	8·5	129
3	60	5·4	5·8	107
4	60	6·2	7·5	121
5	60	6·2	6·4	103
	Mean	6·12	7·08	115

* The differences between the mean of test and control rats are significant (P = 0·01–0·001 after 15 min.; P = 0·02–0·01 after 60 min.), provided the differences between each pair are used as the basis of the calculations.

killed at different time intervals, were employed. Each group consisted of ten rats, five receiving the acid and five acting as controls. As is shown in Table VIII and Fig. 4, the control groups give lower mean values than the test groups. The maximal difference in the second series was similar to that observed in the first series of experiments, where the dose of the acid given to each rat was only 100 mg. compared with 300 mg. given in the second series.

The mean gut weight, in both control and test animals, decreased gradually with time, indicating the gradual adsorption of the intestinal contents. The controls show the same kind of curve as that described by Heller and Smirk (1932): a rapid rate of absorption in the first ½ hr. after which the curve flattens out. In the test group, the same pattern is maintained with two differences; the first part of the curve is less steep and the whole curve is maintained at a higher level throughout.

The weights of the livers did not show any significant difference. Heller and Smirk found the liver to show the most marked increase in weight in response to hydration; in this experiment, however, the increase was not significant.

TABLE VIII

EFFECT OF GLYCYRRHETINIC ACID ON THE ABSORPTION OF WATER FROM THE GUT AND ITS RETENTION IN THE LIVER

Time since Water Load	No. of Rats in Group	Control		Glycyrrhetinic Acid	
		Mean Gut Wt. ± S.E.	Mean Liver Wt. ± S.E.	Mean Gut Wt. ± S.E.	Mean Liver Wt. ± S.E.
15 min.	10	12·6 ± 0·54	4·8 ± 0·41	13·8 ± 0·84	5·1 ± 0·25
30 ,,	10	9·7 ± 0·32	4·6 ± 0·29	12·4 ± 0·67	4·7 ± 0·25
75 ,,	10	9·1 ± 0·43	4·8 ± 0·16	10·2 ± 0·58	4·9 ± 0·1[illegible]

When the rats received 100 mg. of the acid (Table VII), the mean weight of the gastro-intestinal tract was 6.12% of the body weight in the control group 1 hr. after the water load, and 7.08% in the glycyrrhetinic acid group. This is a difference of 0.96% of body weight or 19.2% of the water load.

Heller and Smirk (1932), in their original experiments, showed that the weight of the gut drops in the first hour and reaches a level between 6% and 7% of the body weight and that this level is maintained after that. They reported that absorption of a 5% load is completed 15–20 min. before the height of diuresis is reached. The 19.6% difference between control and glycyrrhetinic acid groups can, therefore, be taken as an estimation of the degree of water retention in the gut caused by 100 mg. of glycyrrhetinic acid.

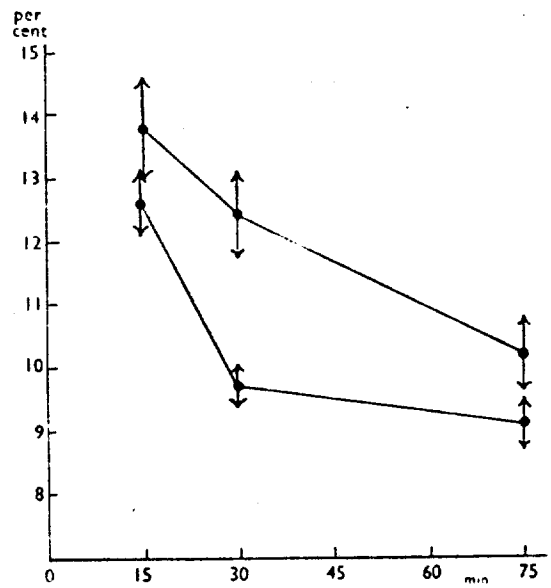

FIG. 4.—Effect of glycyrrhetinic acid on water absorption from the alimentary tract after a water load of 5% of body wt. Each point represents the mean wt. of the gut in five rats. The arrows show the standard error of the mean. Ordinate: Mean gut wt. as percentage of body wt. Abscissa: Time allowed for absorption in min. The upper curve represents the results of rats receiving 100 mg. glycyrrhetinic acid. The lower curve represents the results of control rats receiving the solvent only.

In the diuresis experiments reported in Table IV, the differences between control values and those following 100 mg. of the acid had a mean of 3.04 ml. or 25.3% of the load. Here, however, the load was almost double that used in the absorption experiments. The percentage of water retention in the gut is likely to be smaller with a lower water load if the same dose of the acid is used. Therefore, it is unlikely that the delay in absorption or

water on giving glycyrrhetinic acid by mouth accounts for the whole 25.3% of the water retained.

To investigate further the role played by gastro-intestinal water retention in the anti-diuretic action of glycyrrhetinic acid the following experiment was carried out.

Albino rats (150 g.) were denied food for 24 hr. At 10 a.m. water was withdrawn and the animals divided into pairs of similar weight. At 12 noon, 0.2 ml. propylene glycol was injected intraperitoneally into one rat of each group. The other had 10 mg. of the acid dissolved in 0.2 ml. of propylene glycol. A water load of 12 ml. tap-water was given by stomach tube 10 min. later. The rats were transferred to metabolism cages and urine was collected for 150 min. At the end of the collection the rats were killed and their gastro-intestinal tracts dissected out. The contents of the caecum were emptied out and the gut weighed.

TABLE IX

EFFECT OF GLYCYRRHETINIC ACID ADMINISTERED INTRAPERITONEALLY ON DIURESIS AND WATER ABSORPTION

(Water load 12 ml.)

Group No.	Control		Glycyrrhetinic Acid	
	Urine % of Load*	Gut Weight % of Body Wt.	Urine % of Load*	Gut Weight % of Body Wt.
1	70	7·7	20	7·8
2	30	9·3	2·3	7·9
3	30	7·3	5	8·4
Mean	43	8·1	9·1	8·03

* Urine collection carried out over 150 min.

As shown in Table IX, 10 mg. of the acid intraperitoneally inhibited diuresis in the test rats. The water retained in these rats is not retained in the gut, as is evident by the identical weight of the gut in the control and test rats. In two of the rats, a minor degree of peritoneal effusion was noticed which, however, was not sufficient to account for the water retention.

DISCUSSION

Mineral Metabolism

The success reported by several workers (Groen, Willebrands, and Kamminga, 1951; Groen, Frenkel, and Kamminga, 1952; Card, Mitchell, Strong, Taylor, Tompsett, and Wilson, 1953; and Pelser, Willebrands, Frenkel, Heide, and Groen, 1953) in the treatment of adrenal deficiency with glycyrrhetinic acid and liquorice prompted the investigation of the mode of action of glycyrrhetinic acid on the salt metabolism of rats.

Under experimental conditions in which 1 μg. DOCA caused a significant degree of sodium retention, 400 μg. glycyrrhetinic acid caused no sodium retention, provided the collection of urine was continued long enough to allow urinary flow. A tendency to accelerate potassium excretion was only observed when a sizable potassium load was given.

While it may be justifiable to conclude that glycyrrhetinic acid does not behave like DOCA, as far as the salt metabolism of adrenalectomized rats is concerned, it was conceivable that, like Compounds B, E, or F of Kendall, which are only weak salt retainers, glycyrrhetinic acid may have a beneficial effect on adrenocortical deficiency. No such action on weight or survival time of adrenalectomized rats was, however, found.

Glycyrrhetinic acid, in doses of 3 mg./rat/day, failed to produce any significant change either in the serum sodium or potassium of adrenalectomized rats. This dose is equivalent to 600 mg. to a 60 kg. man. Pelser *et al.* (1953) found 60-100 mg. of the acid to be effective in Addisonian patients when given intramuscularly. Card *et al.* (1953) found 50 mg. doses q.i.d. to be effective in a patient suffering from Addison's disease. The dose employed in the reported rat experiments falls well within the effective range in terms of these human doses.

These findings, therefore, confirm the findings of Nelemans and Stamperius (1949) and Card *et al.* (1953) according to which glycyrrhetinic acid did not cause significant changes in the electrolyte balance or survival times of adrenalectomized rats.

The reported favourable effects of the acid in Addisonian patients in contrast to the lack of such an effect in rats suggest two possible explanations: (*a*) the drug may be metabolized in man in a different way, giving by-products which are active in this respect; or (*b*) the mere water retention in Addisonian patients may have favourable effects under certain conditions.

Water Metabolism

In this series of experiments, it was possible to demonstrate the water-retaining effect of glycyrrhetinic acid in albino rats and in a dog.

In all the previous reports, the water retention by glycyrrhetinic acid has been related to its reported DOCA like effect on mineral metabolism. Since no such effect was produced in adrenalectomized animals, another explanation was called for. It may also be emphasized that in many conditions DOCA has a diuretic, rather than an anti-diuretic, action (Gaunt, Birnie, and Eversole, 1949; Gaunt, 1943; Gellhorn and Ballin, 1946; Winter, 1952

Kuhlman, Ragan, Ferrebee, Atchley, and Loeb, 1939; Winter and Ingram, 1943; Boss, Birnie, and Gaunt, 1949).

The mechanism of the anti-diuretic action of glycyrrhetinic acid appears to be a complex one. No marked change of the blood pressure was observed when the drug was administered intravenously, which suggested that low filtration pressure in the kidneys was not the cause of anti-diuresis. The drug was effective in causing water retention in rats with diabetes insipidus during the height of the diabetic phase. The rate of water retention in these diabetic rats was comparable to the rate of water retention in intact rats under the same conditions. The posterior lobe of the pituitary does not therefore seem to play a role in this mechanism.

The drug, when given by mouth in doses identical with those used in the diuresis experiments, caused a significant degree of water retention in the gut. The percentage of water retained in the diuresis experiments, however, was somewhat higher than the percentage of water retained in the gut. That other mechanisms are involved is shown by the fact that, intraperitoneally, the drug in small doses caused a marked suppression of diuresis and no water retention in the gut. In addition, minute doses administered intravenously to rats anaesthetized with alcohol caused a marked depression of diuresis. In the sodium balance experiments, the drug was observed to cause water retention when administered subcutaneously.

By mouth, glycyrrhetinic acid seems to cause water retention in the alimentary canal as well as in the internal compartments of the body. The second effect may be due to the tubular reabsorption of water in the kidneys as suggested by Molhuysen, Gerbrandy, de Vries, de Jong, Lenstra, Turner, and Borst (1950).

The acid is precipitated in the stomach when introduced in propylene glycol and can still be detected there 4½ hr. after administration. This fact provides a possible explanation of the greater efficacy of parenteral as compared with oral administration.

Summary

1. The reported DOCA-like activity in man of liquorice extracts and glycyrrhetinic acid has been investigated in animals.

2. When these drugs were given to adrenalectomized rats no effect on the sodium balance was observed. Only during the period in which diuresis was suppressed was there a retention of sodium. The increase in potassium excretion was not significant unless the level of the extracellular potassium was highly elevated.

3. Prolonged treatment of adrenalectomized rats with glycyrrhetinic acid caused no significant improvement in the level of serum electrolytes, the body weight, or the survival time.

4. Given by any route, the acid had an anti-diuretic effect. The posterior lobe of the pituitary is not involved in this action of the drug; after parenteral administration, the effect is perhaps produced by a stimulation of the tubular reabsorption of water.

5. When given by mouth, the drug delays water absorption from the alimentary tract. The delay is sufficient to contribute to the water-retaining effect of the drug when administered orally.

6. It is concluded that the actions of the drug are different from those of DOCA.

I wish to express my gratitude to Professor J. H. Gaddum for his continued advice, and to Dr. M. Vogt, whose willing help and guidance made this work possible.

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Production of Experimental Hypertension and Cardiovascular-Renal Lesions With Licorice and Ammoniated Glycyrrhizin

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ABSTRACT

GIRERD, R. J., C. L. RASSAERT, G. DI PASQUALE AND R. L. KROC. *Production of experimental hypertension and cardiovascular-renal lesions with licorice and ammoniated glycyrrhizin.* Am. J. Physiol. 194(2): 241–245. 1958.—The intragastric administration of either licorice or ammoniated glycyrrhizin for 50 days to male rats, unilaterally nephrectomized and given 0.87% sodium chloride solution to drink, caused a blood pressure elevation consistently higher than that induced by DCA under similar conditions. Renal and cardiovascular lesions, consisting essentially of arteriolar necrosis and hyalinization, were very severe in the DCA- and licorice-treated groups, while they were milder in the glycyrrhizin group. Licorice or DCA treatment caused a significant polydipsia and growth impairment, whereas the curves of fluid intake and body growth of the glycyrrhizin treated group remained approximately like those of the controls. These results support some previous reports on the steroid-like activity of licorice. It seems that the hypertensive properties of licorice could be attributed to its glycyrrhizin content, while polydipsia and some other effects may be due to other constituents.

IN 1948 REVERS reported that a preparation containing succus liquiritiae, and which had proved successful in treating patients suffering from peptic ulcer, occasionally caused edema (1). These observations were soon confirmed by Molhuysen *et al.* (2) who found water, sodium and chloride retention, even when no clinical edema was apparent. At the same time the potassium balance was negative and the authors concluded that licorice possesses some deoxycortone-like activity, although the extract used had no therapeutic effect in one case of Addison's disease. However, Groen *et al.* (3) were successful in maintaining several patients with Addison's disease with glycyrrhizinic acid, one principle contained in licorice, while other authors reported that, in bilaterally adrenalectomized patients, glycyrrhizin alone was insufficient, although helpful, for adequate maintenance (4). These negative clinical results have been confirmed experimentally by Galal who reported that neither licorice nor glycyrrhetinic acid had any appreciable effect on adrenalectomized animals (5), and the author concluded that the effects observed were different from those of DCA. However, when ammoniated glycyrrhizin was given together with hydrocortisone to adrenalectomized patients, a synergistic effect was noted (6). The blood pressure elevating effect of licorice in the human being was reported by Harders and Rausch-Stroomann (7).

The purpose of this study was to compare the effects of a crude licorice extract and of ammoniated glycyrrhizin with those produced by DCA, as a result of chronic administration of these materials in the rat.

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MATERIALS AND METHODS

Four groups of 10–19 male Sprague-Dawley rats, which averaged 223 gm on *day 0*, were used as indicated in table 1. Purina dog chow was fed as libitum throughout.

On *day 0*, treatment was initiated and all rats of *group B* were implanted with three 25 mg pure DCA pellets in the interscapular area, while the rats of the other groups were sham implanted.

The powdered oriental licorice extract (MacAndrews and Forbes Co., Camden, N. J.) and the ammoniated glycyrrhizin (MacAndrews

and Forbes) were dissolved in boiling distilled water. The two solutions were administered intragastrically with a soft catheter and a steel gag according to a method already described (8). The daily dosages, per kilogram of body weight, were 10 gm of licorice powder for *group C* and 1 gm of glycyrrhizin for *group D*, each in a volume of 25 ml/kg of body weight. The control rats (*group A*) were subjected to a daily sham gastric catheterization.

The animals were weighed every 3 days. Saline intakes were recorded daily, averaged every 3 days, and expressed as milliliters per 100 gm of body weight per day.

The blood pressures were recorded on all rats between *days* (−) *3* and *0* (pretreatment values) and then on *days 15, 25, 35* and *45*, without anesthesia, by means of a photoelectric tensometer. At least two determinations per animal were made on each of these days, and each determination was the average of at least three successive readings.

All survivors were killed on *day 50* and autopsied. Whenever present, the macroscopic pathologic findings were noted. The organs to be studied were then fixed in SUSA, kept in 95% ethanol, weighed, sectioned, stained with PAS and studied microscopically

The significance of the differences between the group averages was estimated by determining the standard error of the means, and by application of Student's *t* test.

RESULTS

Blood Pressure. The control animals exhibited a moderate rise in pressure to a maximum of 125 mm Hg, while the DCA-treated rats showed a progressive and pronounced rise to a hypertensive level of 178 mm (fig. 1). The glycyrrhizin treated rats were found to have average values which were invariably higher than those of the DCA group, while the averages of the licorice group were the highest of all. On *days 25, 35* and *45*, the differences between each of the three hypertensive groups and the control group were highly significant ($P < 0.001$) but the differences between *groups B, C* and *D* were not. Group comparisons were also made on the basis of averages calculated from the highest determination for each rat, regardless of the day on which it was observed; the values thus obtained were as follows: *group A:* 128, *group B:* 188, *group C:* 186, *group D:* 194.

Saline Intake. Except for a fall in the control values from *day 18* to *30*, both the glycyrrhizin and the control groups exhibited a rather constant and similar intake, averaging between 25 and 30 ml/100 gm/day (fig. 2).

The intake patterns of the animals receiving DCA or licorice remained very similar, except during the last 10 days when the intake of *group B* started to regress; between *days 15* and *50*, the average intake of each of these two groups was found to be significantly larger than that of *group A* ($P < 0.001$) or of *group D* ($P < 0.01$).

Growth. Up to *day 36*, the growth curve of the glycyrrhizin group was similar to that of the controls (fig. 3). The DCA- and the licorice-treated groups also had a normal growth during the 1st week, but started to plateau and lose weight from *day 20*; from that day on, significant differences were observed between these two groups and the controls ($P < 0.001$) or the glycyrrhizin treated group ($P < 0.001$).

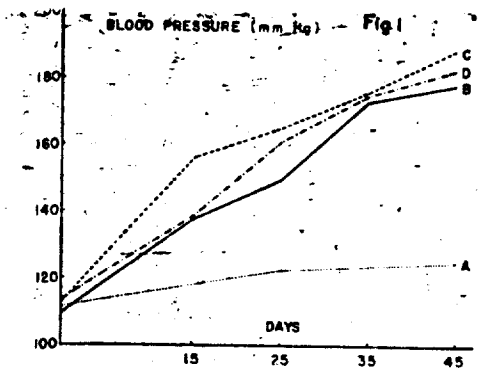
Survival. All control animals survived (10/10). With glycyrrhizin the survival rate was 77% (10/13), whereas with DCA it was 63% (12/19), and only 36% (5/14) with licorice.

Organ Weights. In the three treated groups, the absolute and relative weights of the kidneys and adrenals were higher than the corresponding control weights (table 2). In contrast, the absolute weights of the hypophyses and testes were found to be smaller in the treated groups. On a percentage basis, the heart weights were significantly higher in the three treated groups than in the controls.

TABLE 1. EXPERIMENTAL GROUPS AND THEIR RESPECTIVE TREATMENTS

Group	Individual Treatment	No. of Rats	
		Initial	Final
A	None	10	10
B	DCA, 3 25-mg pellets	19	12
C	Licorice, 10 gm/25 ml/kg/day	14	5
D	Glycyrrhizin, 1 gm/25 ml/kg/day	13	10

The right kidney was removed from all animals on *day* (−) *25* and a 0.87% NaCl solution was given ad libitum from *day* (−) *5* until the end. DCA pellets were implanted and treatment was initiated on *day 0*.



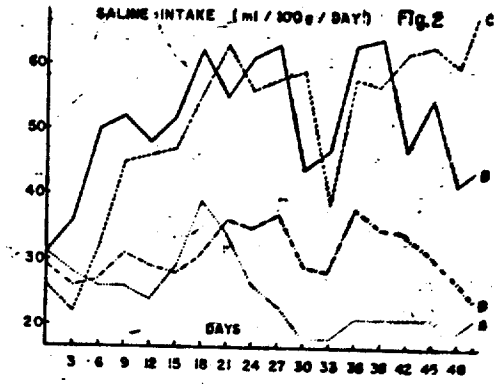


FIG. 1. Effects of various treatments on the blood pressure of unilaterally nephrectomized male rats drinking 0.87% sodium chloride solution. *Group A:* untreated controls; *group B:* DCA treatment; *group C:* licorice treatment; *group D:* glycyrrhizin treatment.

FIG. 2. Effects of various treatments on the fluid intake (0.87% sodium chloride solution) of unilaterally nephrectomized male rats. Groups are the same as indicated in fig. 1.

Tissue Lesions. Table 3 indicates the occurrence of gross pathologic lesions noted at autopsy. Kidney and heart lesions consisted of whitish spots on an irregular organ surface. Small hemorrhagic foci, visible through the pia mater, were considered as brain lesions.

Microscopic examination revealed that the animals of the three treated groups had marked arteriolar necrosis and hyalinization, especially in the kidney, the heart and the testes. These lesions were present in the hearts of the licorice- and glycyrrhizin-treated rats, although these organs had been found macroscopically normal. The kidney was the most severely damaged organ showing intense glomerular hyalinization, tubular dilatation with atrophy of the tubular cells, hyaline casts and proliferation of the interstitial tissue surrounding the glomeruli and tubules. All lesions were very severe in the DCA- and licorice-treated animals; they were milder in the glycyrrhizin group. No lesions were found among the control animals.

DISCUSSION

The results confirm experimentally the clinical observations of Harders and Rausch-Stroomann (7) who noted a blood pressure elevation in patients treated with licorice. With the dosage levels employed in this study, it was found that both glycyrrhizin and licorice caused a blood pressure elevation which was consistently, although not significantly, higher than that induced by DCA. The relatively high doses of both compounds did not seem to cause any gastrointestinal or systemic disturbances aside from those classically induced by DCA. The dose of licorice was 10 times that of glycyrrhizin but, since licorice contains between 6 and 14% of glycyrrhizin (9), the amount of glycyrrhizin contained in both groups was about the same. Therefore, it may be assumed that the hypertensive properties of licorice are due in part, if not fully, to glycyrrhizin. It is interesting to note that, although the blood pressures showed identical trends in the three treated groups, licorice and DCA induced similar polydipsia and inhibition of growth, while glycyrrhizin treatment caused little change in saline intake and body weight, when compared to the controls. This dissociation between blood pressure and intake observed with glycyrrhizin is interesting in view of the observations of Green *et al.* (10) con-

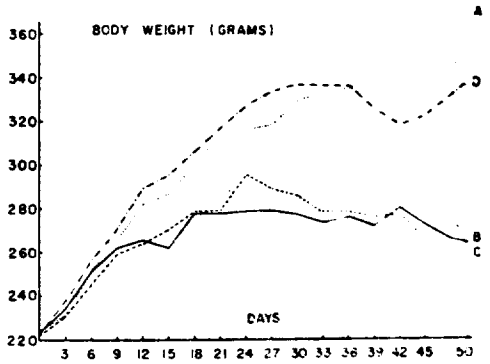


FIG. 3. Effect of various treatments on the body weight of unilaterally nephrectomized male rats drinking 0.87% sodium chloride solution. Groups are the same as indicated in fig. 1.

TABLE 2. ORGAN WEIGHT AVERAGES PER GROUP AND SIGNIFICANCE OF THEIR DIFFERENCES

Groups	Av. Body Wt. at Autopsy, gm	Organ Weights									
		Kidney		Heart		Adrenals		Pituitary		Testes	
		mg	mg %	mg	mg %	mg	mg %	mg	mg %	mg	mg %
A	370	2268	613	1074	290	44	12	8.3	2.2	2831	768
B	264	2732	1038	1186	457	52	20	6.2	2.4	2081	781
C	262	2459	1028	1037	441	55	24	5.6	2.3	2246	1004
D	358	2709	847	1203	376	51	17	6.6	2.1	2502	786
A-B		<0.001	<0.001	<0.05	<0.001	<0.01	<0.001	<0.01	<0.4	<0.001	<0.8
A-C		<0.4	<0.001	<0.7	<0.001	<0.01	<0.001	<0.001	<0.7	<0.01	<0.001
A-D		<0.05	<0.001	<0.1	<0.001	<0.05	<0.02	<0.01	<0.2	<0.2	<0.4

P values between the controls (*group A*) and the treated groups are indicated at the bottom of each column.

cerning the relationship between sodium intake and blood pressure elevation. In our study, the highest hypertensive levels have been reached with glycyrrhizin without an intake increase equivalent to the one reported by these authors. This would be indicative of differences in the mode of action of glycyrrhizin as compared to DCA and licorice, or it may mean that one, or perhaps several, constituents of licorice other than glycyrrhizin has a pronounced polydipsic effect. For instance, estrogenic substances have been reported to occur in licorice (11) and it can be assumed that they may have had an effect upon water and sodium metabolism related to that reported for various estrogens (12).

The cardiovascular and renal lesions were qualitatively very similar for the three compounds, suggesting a DCA-like action. The reason that they occurred generally less frequently and were not as severe with ammoniated glycyrrhizin is probably more related to the reduced sodium intake than to the presence, in negligible amount, of dietary ammonium ions attached to glycyrrhizin and which have been shown to exert a protective effect on the cardiovascular system under certain conditions (13).

TABLE 3. GROSS PATHOLOGY OF THE SURVIVING ANIMALS AT AUTOPSY

Treatment	*Group A*	*Group B*	*Group C*	*Group D*
	Controls	DCA	Licorice	Glycyrrhizin
Survivors	10	14	5	11
Kidney	0	14	4	8
Heart	0	4	0	0
Brain	0	7	4	3
Mesenteric periarteritis nodosa	0	2	3	1
Ascites	0	2	1	2

Each figure represents the number of rats presenting lesions of the indicated organ or type.

The occurrence of ascites in the three treated groups may be regarded as indirect evidence and further support of the fact that all three compounds had some sodium-retaining properties. It also confirms some recent observations concerning the desoxycorticosterone-mimetic action of ammoniated glycyrrhizin (14). These observations, together with the effects upon blood pressure, the occurrence of periarteritis nodosa and the adrenal hypertrophy, are reminiscent of a mineralocorticoid-like effect.

Since the oral doses given were very large it is likely that only a fraction thereof was absorbed by the animals; smaller doses may therefore be just as effective and could easily be incorporated in the food. More studies are now being undertaken to find out if this approach could lead to a practical method to produce hypertensive animals, and to determine if licorice or glycyrrhizin-induced hypertension persists after cessation of treatment, as is known to be the case in corticoid-induced hypertension.

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ANTIARTHRITIC ACTIVITY OF GLYCYRRHIZIN IN ADRENALECTOMISED RATS

M.L. Gujral, K. Sareen, D. P. Phukan[1] and M.K.P. Amma

Recently much attention has been paid to *Glycyrrhiza glabra* because of the structural resemblance of its main constituent, glycyrrhizin, to adrenocorticoids (Gujral *et al*[1]). Glycyrrhizin has been known to possess DOCA-like properties such as retention of sodium and water and excretion of potassium (Molhuysen *et al*;[2] Louis and Conn[3]). Gujral *et al*[1] reported the antiarthritic and anti-inflammatory action of the diglucuronide glycyrrhizin, $C_{42}H_{62}O_{16}$, in Brownlee's formaldehyde-induced arthritis in non-adrenalectomised rats. Other investigators (Finney and Somers[4]) have concentrated their attention on the antiarthritic activity of its triterpenoid "aglycone" glycyrrhetic acid, $C_{30}H_{46}O_4$. The present investigation deals with the study of the antiarthritic and anti-inflammatory effect of glycyrrhizin in adrenalectomised rats to elucidate its mode of action and to find out whether it is mediated through the pituitary-adrenal axis or has an independent action of its own.

In the present study attention has been directed mainly to glycyrrhizin rather than to glycyrrhetic acid because of the easier and cheaper isolation of the former in good yield from the plant. The hydrolysis of the diglucuronide to its aglycone is invariably accompanied by partial inactivation through stereochemical changes which decreases the yield of the active material and also adds to the cost of the final product. Only the β-isomer of glycyrrhetic acid has been reported to be active (Finney and Somers).[4] As there is not much difference between the activities of the two compounds, glycyrrhizin has been preferred.

MATERIAL AND METHOD

Albino rats of the same strain, colony and age group weighing 90 ± 10 G. were selected. They were given sodium chloride and glucose previous to the day of operation. After ether anesthesia to the animals, one-stage adrenalectomy was performed by a method similar to that of Schultzer.[5] Post-operatively 2.5 ml. of dextrose-saline (sodium chloride 1% + dextrose 5%) was injected subcutaneously into the abdomen and repeated again in the afternoon. In each case sufficient amount of sodium chloride and glucose was added along with food and water. In the bilaterally adrenalectomised rats arthritis was produced by the method of Selye[6] and Brownlee.[7] The animals were divided into groups of six rats each, keeping the mean linear cross section immediately below the ankle joints within 0.1 mm. of the control group. On the second day after adrenalectomy, 0.1 ml. of a 2 per cent formaldehyde solution was injected into each limb of the rat just beneath the plantar aponeurosis. The injections were repeated on the ninth day of adrenalectomy. The drug to be tested was suspended in 3% gum acacia and fed orally by a gastric canula daily for ten days from the commencement of the experiment. By means of a micrometer screw gauge the linear

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[2] This study formed a part of the thesis submitted by D. P. Phukan in November 1959, for M. D. (Pharmacology) Degree of Lucknow University.

cross section immediately below the ankle joints of both hind limbs were measured and day to day changes in size were recorded for each group for a period of ten days. The mean value for all the observations with their standard errors derived from the group means and t and P values were calculated.

Two series of experiments were performed, one consisting of three groups and the other of two groups of adrenalectomised rats each group containing six animals. The groups were divided as follows:

First series.—

Group I. DOCA. 250 μg./100 G. body weight, subcutaneously, daily.

Group II. DOCA. 250 μg./100 G., subcutaneously+hydrocortisone, 0.5 mg./100 G., orally daily.

Group III. DOCA. 250 μg./100 G., subcutaneously+glycyrrhizin, 20 mg./100 G., orally, daily.

Second series.—

Group I. Hydrocortisone. 0.5 mg./100 G., orally, daily.

Group II. Glycyrrhizin. 20 mg./100 G.,+hydrocortisone, 0.5 mg./100 G., orally daily.

RESULTS

The first injection of 0.1 ml. of 2 per cent formaldehyde produced hyperemia and edema, especially in the ankle regions. However, the edema and hyperemia were more pronounced and quite differentiated after the second injection of formaldehyde (4th day after adrenalectomy) (see figures 1, 2 and 3).

The results of the first series of experiments, in which the effect of DOCA, DOCA + hydrocortisone and DOCA + glycyrrhizin has been studied, are given in table I and figure 4.

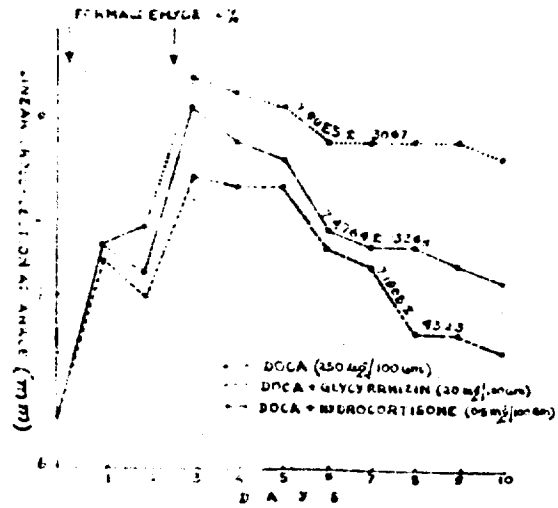

Fig. 1.

TABLE 1. *Antiarthritic Effect of DOCA, DOCA + Hydrocortisone and DOCA + Glycyrrhizin in Adrenalectomised Rats.*

No.	Drug	Mean diameter (mm.)	S.D.	S.E. of mean	t	P
1.	DOCA, 250 μg./100 G.	7.9035	± 0.3067	± 0.097	..	—
2.	DOCA, 250 μg./100 G. + hydrocortisone 0.5 mg./100 G.	7.1965	± 0.4323	± 0.1368	5.156	<0.001
3.	DOCA, 250 μg./100 G. + glycyrrhizin, 20 mg./100 G.	7.4764	± 0.3364	± 0.1064	3.1303	<0.010

It is revealed that glycyrrhizin possessed a significant antiarthritic activity in adrenalectomised rats ($P < 0.01$) in comparison to hydrocortisone as reference standard ($P < 0.001$).

In the second series a combination of hydrocortisone + glycyrrhizin was studied. The results of these experiments are given in table 2 and figure 5.

FIG. 5.

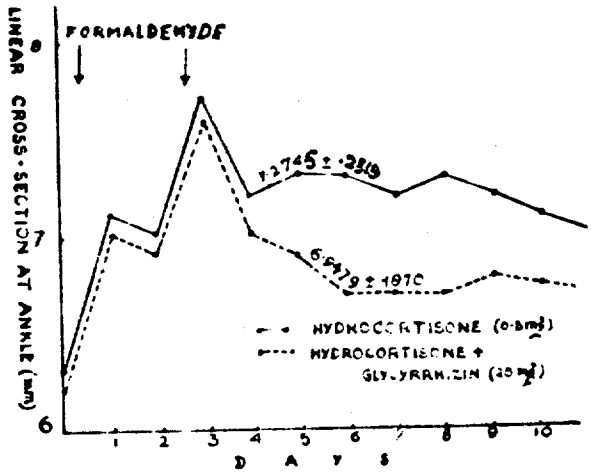

TABLE 2. *Antiarthritic Effect of Hydrocortisone and Hydrocortisone + Glycyrrhizin in Adrenalectomised Rats.*

No.	Drug	Mean diameter (mm.)	S.D.	S.E. of mean	t	P
1.	Hydrocortisone, 0.5 mg./100 G.	7.2745	± 0.2318	0.0733	-	--
2.	Hydrocortisone, 0.5 mg./100 G. + glycyrrhizin, 20 mg./100 G.	6.9479	± 0.1870	0.0591	3.4589	0.005 – 0.001

It is evident that the combination of glycyrrhizin and hydrocortisone, in the doses mentioned, had a greater activity ($P < 0.005$) than that of hydrocortisone alone, showing thereby that glycyrrhizin potentiated the action of hydrocortisone.

DISCUSSION

In the production of arthritis in adrenalectomised rats formaldehyde was injected according to Selye's[10] original method of injecting on 2nd and 4th day after adrenalectomy. There was a slight difference in the technique used in the present study from that used by Brownlee.[8] Brownlee injected formaldehyde on the 5th and 7th day after adrenalectomy. His idea was to wait for four days in order to exhaust all the circulating endocrinal hormones. As the experiments here have been performed at a higher room temperature, it was not possible to keep the animals alive only on dextrose-saline for four days without giving DOCA or hydrocortisone. For this reason the animals were kept alive for the first two days only on dextrose-saline on the assumption that all the remaining circulating hormones would be exhausted in this period. After this a minimum dose of DOCA, 250 μg. 100 G. subcutaneously, was used in these studies to keep the animals alive during the duration of the experiment.

There are several controversial reports regarding the use of DOCA in adrenalectomised animals. Some of the observers reported that DOCA over-dosage caused adrenalectomised rats to develop arthritis more frequently in cold conditions than intact animals (Hall, Selye *et al*[illegible]). Selye[illegible] used a dose of 5 mg. DOCA daily in his study of formaldehyde-induced arthritis in adrenalectomised rats. However, 1 mg. DOCA daily subcutaneously did not exacerbate formaldehyde-induced arthritis in adrenalectomised rats (Brownlee[8]). Besides, Brownlee also claimed beneficial effects with 1 mg. of DOCA plus 20 mg. ascorbic acid daily in normal as well as adrenalectomised rats. Gujral and Saxena could not confirm his results in normal rats. As the same dose of DOCA was given to both the control and the drug treated animals, it cannot interfere with the results presented here. Hence the amount of exacerbation or reduction of inflammatory responses by DOCA in the minimal dose would be the same in all the groups.

Glycyrrhizin, 20 mg. 100 G. orally, has been found to be effective in formaldehyde-induced arthritis in adrenalectomised rats. As will be evident from table 1 the effect of glycyrrhizin (20 mg. 100 G., orally) is slightly less than that of hydrocortisone (0.5 mg. 100 G., orally) in adrenalectomised rats. In normal animals, however, the effect of this dose of glycyrrhizin is equal to that of 0.5 mg. of hydrocortisone (Gujral *et al*[illegible]). These findings strongly suggest that the action of glycyrrhizin is not mediated through the pituitary-adrenal system.

Glycyrrhizin 20 mg. 100 G. + hydrocortisone 0.5 mg. 100 G. orally is more effective than hydrocortisone (0.5 mg. 100 G.) alone (table 2). This indicates that glycyrrhizin potentiates the action of hydrocortisone. This might also explain a slight decrease in the potency of glycyrrhizin in adrenalectomised rats.

These findings are in agreement with those of Borst *et al*, Hart[9] and Hudson *et al*[illegible] who found the potentiating action of *Glycyrrhiza glabra* and glycyrrhetic acid in Addison's disease and other diseases [illegible] glands. Hydrocortisone is known to have a beneficial [illegible] arthritis in man (Hench *et al*).[illegible] It has also [illegible] against formaldehyde-induced arthritis in adrenalectomised [illegible] normal rats (Selye).[illegible] It can, therefore, be strongly [illegible] that glycyrrhizin should be given a fair trial in early cases [illegible] arthritis and other inflammatory conditions along with hydrocortisone.

SUMMARY

1. The antiarthritic and anti-inflammatory effect of glycyrrhizin in Brownlee's formaldehyde-induced arthritis in adrenalectomised rats has been studied.
2. As this effect of glycyrrhizin can also be demonstrated in adrenalectomised rats, its action does not appear to be mediated through the pituitary-adrenal axis. Its action may probably be peripheral.
3. Glycyrrhizin potentiates the antiarthritic action of hydrocortisone in rats. The combination, in doses mentioned, produces better results than hydrocortisone alone.

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ENDOCRINOLOGICAL STUDIES ON GLYCYRRHIZIN IN RATS*

M.L. Gujral, K. Sareen, D.P. Phukan** and M.K.P. Amma

In a previous communication Gujral *et al*[5] reported the anti-arthritic and anti-inflammatory activity of glycyrrhizin, $C_{42}H_{62}O_{16}$, the active principle of *Glycyrrhiza glabra*, in normal and adrenalectomised rats. In order to elucidate its mode of action it was decided to pursue these studies further to investigate the endocrinological response, if any, to this diglucuronide. Similar studies have been reported on crude glycyrrhiza extract (Card *et al*[1]) and on the "aglycone" glycyrrhetic acid, $C_{30}H_{46}O_4$, (Card *et al*,[1] Finney *et al*,[2] Finney and Tarnoky,[3] Galal,[4] Karus *et al*[8],[9] and Wenzel and Emick[10]).

MATERIAL AND METHOD

1. Survival time in adrenalectomised rats.—36 male rats of the same age group and weighing 90 ± 10 G. were divided into three groups so that the mean weight of each group was approximately the same. Bilateral adrenalectomy was performed according to the method of Schultzer[11] and the drug, in the form of a suspension in gum acacia, was administered as follows:

Group I—Control (untreated).

Group II—Dextrose-saline, 2.5 ml., subcutaneously (abdomen).

Group III—Dextrose-saline, 2.5 ml., subcutaneously+glycyrrhizin, 40 mg./100 G. orally.

Dextrose saline consisted of dextrose 5% + sodium chloride 1%. The animals were kept at a temperature of 33 ± 1°C to serve as a source of stress, a substitute for Selye's cold-stress technique.[14] The survival time of each animal was noted and the results were analysed statistically.

2. Endocrinological studies.—36 albino rats weighing 90 ± 10 G. were divided into four groups. The first group, consisting of 12 animals as control, received only gum acacia, 40 mg./100 G. body weight. The remaining three groups, consisting of 12, 6 and 6 animals, received orally 40, 80 and 120 mg./100 G. glycyrrhizin respectively.

(a) *Weight of the adrenals and the spleens.*—The animals were sacrificed five hours after the administration of the drug and their adrenals and the spleens were carefully dissected out and weighed. The weights were expressed in terms of absolute (real) weight in mg. and relative weight in mg./100 G. body weight.

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** This study formed a part of the thesis submitted by D. P. Phukan in November, 1959, for M. D. (Pharmacology) degree of Lucknow University.
Received for publication on 2nd January, 1961.

(b) *Adrenal ascorbic acid and cholesterol levels.*—The left adrenal was processed for the estimation of ascorbic acid according to the method of Karkun *et al*[7] and the right one for that of cholesterol by the method of Roy *et al.*[8] The results were expressed in the case of ascorbic acid in mg./100 G. and in that of cholesterol in mg./G. of the adrenal gland.

3. Hematological studies.—Two series of experiments were performed for studying the effect of glycyrrhizin on total white blood cells, lymphocyte and eosinophil count in non-adrenalectomised and adrenalectomised rats.

In the first series, 30 non-adrenalectomised rats weighing 90 ± 10 G. were selected and divided into three groups as follows:

Group I—consisted of 12 rats (control) which received gum acacia (vehicle), 40 mg./100 G., orally.

Group II—consisted of 12 rats which received glycyrrhizin, 40 mg./100 G., orally.

Group III—consisted of 6 rats which received glycyrrhizin, 80 mg. orally.

In the second series 18 bilaterally adrenalectomised male rats weighing 90 ± 10 G. were divided into three groups of six rats each.

Group I—control (vehicle) treated.

Group II—received glycyrrhizin, 40 mg./100 G., orally.

Group III—received glycyrrhizin, 80 mg./100 G., orally.

RESULTS AND DISCUSSION

The results of the above studies have been recorded in tables 1, 2 and 3. Glycyrrhizin does not prolong the mean survival time of adrenalectomised rats. Both dextrose saline and its combination with glycyrrhizin, at the dose levels mentioned, significantly prolong the mean survival period at a temperature of 33 ± 1°C ($P < 0.02$ and 0.005 - 0.001) as compared to that of the control (table 1). The effect of dextrose-saline is not in any way enhanced by the addition of glycyrrhizin indicating that the latter has no action of its own in this respect ($P > 0.9$). It, therefore, has no DOCA- or glucocorticoid-like action in prolonging the lives of the adrenalectomised rats. These observations are in conformity with those obtained with crude glycyrrhiza extract[1] and with glycyrrhetic acid.[1,2,3,10]

Even upto 120 mg./100 G. dose, which is 12 times the minimum antiarthritic dose in non-adrenalectomised rats, glycyrrhizin has also no significant effect on the absolute or relative weights of the adrenal glands (table 2A) or on their ascorbic acid and the cholesterol content (table 2B), which shows the absence of any glucocorticoid- or ACTH-like activity. These confirm the observations of Louis and Conn,[11] Kraus *et al*[5,9] and Linko and Vasama[10] in human trials regarding the mild inhibitory action of ammonium glycyrrhizinate on the endogenous production of ACTH as indicated by a consistent decrease in the excretion of 17-ketosteroids. The effect of its aglycone, glycyrrhetic acid, on the adrenal cortex of rat is not yet clear. Linko and Vasama,[10] from their histological studies found that it had a DOCA-like effect on the zona glomerulosa and on the transitional zone. Finney and Somers,[2] however, did not

TABLE I : *Effect of Dextrose-saline and Dextrose-saline + Glycyrrhizin on the Mean Survival Time of Adrenalectomised Rats.*

Drug	Mean survival time (hr)	S.D.	S.E.	t	P
1. Control	24.33	± 16.51	± 4.77	....	....
2. Dextrose-saline	69.66	± 52.55	± 16.05	2.702	<0.02
3. Dextrose-saline + glycyrrhizin	73.90	± 47.16	± 13.63	3.431	0.005–0.001

TABLE IIA : *Absolute and Relative Weight of the Adrenals and the Spleens*

Group	No. of rats	Drug	Dose mg/100 G. body weight	ADRENALS Absolute weight (mg.) Mean	S.E.	P	ADRENALS Relative weight (mg./100 G) Mean	S.E.	P	SPLEEN Absolute weight (mg.) Mean	S.E.	P	SPLEEN Relative weight (mg/100 G) Mean	S.E.	P
I.	12	(vehicle)	40	17.17	± 0.73	..	18.88	± 0.61	..	557.3	±25.9	..	623.58	±23.87	..
II.	12	Glycyrrhizin	40	16.17	± 0.88	<0.4	17.93	± 0.83	<0.4	548.1	±28.9	<0.8	608.4	±31.22	<0.7
III.	6	—do—	80	15.73	± 1.1	<0.3	17.47	± 2.07	<0.3	564.3	±47.9	<0.9	596.19	±57.63	<0.7
IV	6	—do—	120	15.31	± 1.05	<0.2	17.03	± 1.27	<0.25	527.3	±42.2	<0.6	588.83	±51.90	<0.6

TABLE IIB: *Adrenal Ascorbic Acid and Cholesterol Levels*

Group	No. of rats.	Drug	Dose mg./100G body weight	Ascorbic Acid (mg./100 G. of adrenal tissue) Mean	S.E.	P	Cholesterol (mg./100 G. of adrenal tissue) Mean	S.E.	P
I. (Control)	12	Vehicle	40	430.25	± 13.91	....	29.23	± 1.76	....
II.	12	Glycyrrhizin	40	445.46	± 15.16	<0.5	30.12	± 1.67	<0.8
III.	6	do	80	483.00	± 29.85	<0.2	31.42	± 1.66	<0.4
IV.	6	do	120	509.50	± 36.70	<0.1	33.35	± 1.40	<0.1

TABLE IIIA: *Effect of Glycyrrhizin on the Hematological Values of Non-adrenalectomised Rats.*

Group	Treatment	Morning count: Mean Total W.B.C. per cmm. of blood (with S.E.)	Mean Total Lymphocytes per cmm. of blood per (with S.E.)	Mean Total Eosinophils cmm. of blood (with S.E.)	Evening count: Mean Total W.B.C. per cmm. of blood (with S.E.)	Mean Total Lymphocytes per cmm. of blood (with S.E.)	Mean Total Eosinophils per c.mm. of blood (with S.E.)
I.	Control	[illegible]	6237.1	[illegible]	11837.5	6616.58	566.6
		[illegible]	±372.2	65.8	±453.4	±346.2	±52.4
II.	Glycyrrhizin 40 mg./100G. orally	10308.[illegible]	5729.2	445.8	11141.1	5580.6	408.3
		[illegible]	442.2	±48.5	±583.8	±476.8	±43.5
III.	Glycyrrhizin 80 mg 100 G. orally	11[illegible].3	6926	500	12666.6	5910.6	441.6
		761.76	±693.59	± 41.9	±642	±698.45	± 66.2

TABLE IIIB : *Effect of Glycyrrhizin on the Hematological Values of Adrenalectomised Rats.*

Group	Treatment	Morning count			Evening count		
		Mean Total W.B.C. per cmm. of blood (with S.E.)	Mean Total Lymphocytes per cmm. of blood per (with S.E.)	Mean Total Eosinophils cmm. of blood (with S.E.)	Mean Total W.B.C. per cmm. of blood (with S.E.)	Mean Total Lymphocytes per cmm. of blood per (with S.E.)	Mean Total Eosinophiles c.mm.of blood (with S.E.)
I.	Control	9400 ±531.4	5100 ±517.5	550 ±68.	10266.6 ±553.6	5092.6 ±419.2	516.4 ± 44.9
II.	Glycyrrhizin 40 mg./100 G. orally	9766.6 ±449.8	5630.6 ±501.2	558.3 ± 65	9800 ±629.4	4883.3 ±385.3	500 ± 46.4
III.	Glycyrrhizin 80 mg./100G. orally	9100 ±329.3	5192 ±409.8	483.3 ±47.7	10133 ±448.6	4452.6 ±386.9	375 ± 40.9

find much histological changes. There was no adrenal atrophy and sections of the glands were normal except for a slight thinning of the lipid in the zona glomerulosa which was far less severe than that caused by DOCA.

Similarly, in doses upto 120 mg./100 G., it did not cause any significant change in the absolute or relative weight of the spleens (table 2A) nor did it affect the total W.B.C., lymphocyte and absolute eosinophil count after five hours of the drug treatment, both in normal and adrenalectomised rats (Table 3A and B). If it had possessed any ACTH- or glucocorticoid-like action, it should have caused cosinopenia and lymphocytopenia. Moihuysen *et al*[12] also did not observe any significant change in the eosinophil count in healthy subjects even with very heavy doses of the dried aqueous extract of *Glycyrrhiza glabra*. Similarly, Louis and Conn[11] observed no change in the level of circulating eosinophil by using ammonium glycyrrhizinate in human trials.

SUMMARY

1. Glycyrrhizin, like crude glycyrrhiza extract and the aglycone glycyrrhetic acid, does not prolong the survival time of adrenalectomised rats. It has, therefore, no DOCA- or glucocorticoid-like action in these animals at the dose levels mentioned.

2. It also does not cause any depletion of the ascorbic acid and the cholesterol levels of the adrenal gland or any change in their absolute or relative weights. It seems to have no effect on the total white blood cells, circulating lymphocyte and the eosinophil count or on the absolute or the relative weight of the spleens. It does not, therefore, possess any glucocorticoid- or ACTH-like activity.

3. It may have an action of its own in eliciting the antiarthritic and anti-inflammatory effect.

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FOR PRACTICE

From the 1. Medical University Clinic, Hamburg-Eppendorf
(Director: Prof. Dr. H.H. BERG)

High pressure caused by chronic liquorice poisoning

A warning against uncontrolled self-treatment

By Medical Doctors H. HARDERS and J.G. RAUSCH-STROOMANN

For years, the readers of illustrated magazines and daily papers have been exposed to professional medical problems; serious objections must be raised against the above. Almost no issue of the well-known illustrated magazines is without an article which extols either inadequately tested therapeutic methods, representing them as proven panacea, or which praises earlier methods as the latest achievements.

The danger shall be shown here which result from the constantly recurring confrontation of medical laymen with inexpertly presented professional problems.

In the first place, reference should be made to the unjustified hopes of the chronically ill, and especially of cancer patients. raised by reports on wonder drugs. This merely leads to loss of time needed for professional treatment; cash is spent for ussless procedures, and the hope of the ailing once more changes into despair.

Furthermore, the discussion of many chronic diseases generates a general dread of diseases without--apart from a few exceptions--providing any information for the non-professional public which could be defined as valuable.

The popularization of medical problems also affects the relationship between physician and pateient most adversely . insofar as the patient, on the basis of his "knowledge", follows steps taken by the physician with critical eyes, and discusses them at home.

The "medical information" of the public, however, involves another risk, i.e. uncontrolled self-treatment with the recommended remedies. The subject will be discussed here on the basis of a case observed by us.

Our patient had taken liquorice for months, as suggested by an illustrated magazine article, on account of an ulcus ailment diagnosed years earlier. He then was admitted to the clinic with high hypertonicity and stenocardiac complaints.

We believe that this brief report is justified with reference to the dangers inherent in the popularization of medical problems in general, and with reference to liquorice therapy in particular.

Case: H.K., 48 years old, farmer. Family data: father died at age 72 following a heart disease; mother suffering from migraine.

E.A. : does not recall childhood diseases. From 1939 to 1943, periodical rythmic upper abdomen complaints, with peaks in Spring and Fall. Incompatibility with legumens, cabbage and (black) rye bread. Pain at night and when fasting. Relief through intake of food. In 1943 ulcus duodeni and hyperacidity diagnosed during a test. Therapy: ulcus therapy. Since that time, minor complaints only. Since the beginning of 1951, minor motion dyspnoe, shooting pains in the cardiac area, occasional dizzy sensation. Improvement due to nitrogen preparations. In March 1951 RR. measured 140 and 145 systolic on the occasion of a tooth repair. Since April 1952 he ate a finger-thick liquorice bar each 2nd day on account of earlier ulcus complaints, on the basis of a newpaper article, without medical prescription or control. Weight of bar: 35 g, i.e. a total of approximately 4000g! Since then increased cardiac sensations, at night "agitation" in the heart area, throbbing of the pulse at the temples, feeling of oppression. By the middle of Sept. 1952 the family doctor who knew nothing about the liquorice medication, determined a systolic blood pressure of 230 and prescribed Hydergin.

Diagnose: body length 179 cm, body weight 64,5 kg. A somewhat gaunt, well-muscled man with nasolabial wrinkles, indication of arcus senilis, myogeloses, no evident edemas, no icterus, glands without symptoms. Respiratory system: hypersonorous head resonance, low AG, no side tones.
Circulatory system: RR 240/120, regular, tones pure. 2. AT. not emphatic, pulse strained, frequency 88/min. Foot pulses +.

Digestive system: full upper jaw denture, partial lower jaw denture. 6 remaining teeth. Tongue somewhat furry, throat and tonsils without symptoms, liver and spleen not enlarged. Abdominal walls well-muscled.

Rectal: prostate moderately large, rough.
Neurologic diagnosis: normal.

Other examinations: blood count: hb. 81%, 3,9 mill. ery.., F.I. 1,05. 7200 white (blood cells). Differentially: not conspicuous. BSG 4/10 mm. cholesterin 241 mg%, potassium 20,8 mg%, sodium 310 mg%. Wa R. neg., sugar neg., urobiligonen cold neg., warm pos. Sediment: sporadic leucocytes, epitheliums. Urine culture: staphylo- and streptococci. Spontaneous urine concentration max. 1021. Salt excretion inconspicuous. Kidney function test according to VOLHARD: supply 1500 ccm. Urine volume during the first 4 hours: 1200 ccm; during the subsequent 20 hours: 1200 ccm. Maximal concentration 1022.
ECG: no pathological findings.

Ophtalmological (Dr. H. PUNDER, Eye Clinic of the University): papillae and arteries regular, yellowish reflections, veins well-filled, no signs of intersecting, no foci, no bleeding.
Evaluation: no pathological diagnosis for the background of the eyes.
X-ray examination: no pathologic findings at the thorax organs. Smooth esophagus passage, stomach, bulbus and upper small intestine : no (pathological) diagnosis . No indication of stone.
Sequel: after omission of the liquorice, with a salt-free diet, bed rest, mild laxative and sedation therapy the initially very high blood pressure decreased slowly and constantly, returning to normal values within 14 days.
Chart:

The complaints disappeared completely at the same time, without any additional therapy. During the ambulant control 3 months later a blood presure of 145/95 was measured, i.e. identical to the value already established in March 1951. Subsequ ent ECG-controls showed various findings indicating a myocardiac lesion. Cholesterin control: 180 mg%.

Epicrisis and discussion: it is evident that a high hypertonicity was present, which obviously developed during the intake of the excessive dosages of liquorice continued for months; it was lastingly reduced to the earlier known normal values after discontinuation (of the medication). Clinical, X-ray and ophtalmological diagnoses coincide with the above, insofar as they indicate no evidence of an earlier hypertonicity present for a prolonged period of time. It can be assumed that the minor coronay complaint which was present earlier was considerably intensified by blood pressure changes and changes in the water- and mineral circulation systems (hydremia, potassium deficiency); it can likewise be assumed that the blood cholesterin values increased considerably; they decreased after restriction of the liquirice consumption. It is admittedly possible, according to subsequent ECG-controls, that a myocardiac lesion could have resulted from the chronic intoxication, or that it could have been present earlier. No after-effects of the previously diagnosed ulcus condition, which had prompted the self-treatment at issue, could be detected.

Data on side effects during therapy with succus liquiritiae were supplied as early as 1946 by REVERS who was firts to report on said therapy. He specifies edemas, headaches and sensations of oppression in more than 20% of his cases. A thorough analysis of the function mechanism of succus liquiritiae was carried out by BORST and collaborators, MOLHUYSEN, GERBRANDY, de VRIES, de JONG, LENSTRA and TURNER. These authors confirm the data of REVERS concerning the side effects; they found latent or manifest edemas with salt concentration and potassium loss, as well as vein- and blood pressure increases. Said authors therefore likened the effect of orally administered liquorice extract to DOCA. They make specific reference to the

risks for the myocardiac resulting from rising blood pressure and potassium deficiency. The findings of BORST and collaborators explain the earlier observations of van den BERGHE and PANNEKOEK concerning edemas and asthma cardiale in the course of liquorice therapy or during the continued consumption of candies containing liquorice. SCHULZE and FRANKE found in 1951 in half of their ulcus patients treated with comparatively high doses of succus liquiritae edemas, salt retention and in part of the cases, a considerable blood pressure increase; thereby at first only the systolic value rose when the amplitude increased. The diastolic value rose only in the course of the further therapy. In 1950 and 1951 CAYLEY reported on three and STRONG on two cases of potassium deficiency with the corresponding clinical and ECG diagnoses, in the course of lung tuberculosis therapy with PAS, whereby succus liquiritiae was added as taste improvement. ROUSSAK even reported on a case with lethal outcome in 1952, involving electrolyte shift interpreted as hypo-potassic (hypokaliämisch) alkalosis with tetany during the PAS therapy of a tubercular meningitis case (15-year-old girl). In this case liquorice juice was likewise added as (taste) corrigendum. The patient died one month before the BORST paper on the function mechanism of succus liquiritiae was published. HENNEMANN, STAAMANN, BAUMGARTEN and ALBERT also found blood pressure increases in their cases, which receded within a week after the discontinuation of the remedy. But no values as extreme as those reported by SCHULZE and FRANKE could be determined for the above, evidently because the dosage rate was lower. A good observation, which corresponds to a parallel case reported by us, originates from KERN. The unusual case at issue concerns a pharmacist who had taken succus liquiritiae during 9 days on account of supposed stomach ailments; he complained about a swollen face, undefined discomfort and stenocardia. A hypertone of 185/120 was found, as well as a pathological ECG and a micro-hematuria. After the discontinuation of the medication the blood pressure was reduced to 135/100 and the complaints soon disappreared. KERN is correct when

voicing concern regarding a massive use of succus liquiritiae in case of older ulcus patients because the danger of an adverse effect on a latent coronary condition exists. KLIMPER found in the course of ulcus therapy with succus liquiritiae a well-defined hyper-lipaemia (Hyperlipämie), and beginning from the 3rd treatment week, changes in the standing ECG. ARGELANDER also detected a cholesterin increase and in 2 cases out of 13 patients, a condiserable blood pressure increase or edema. The last-mentioned author demands precise dosage and medical control, stressing the dangers of propaganda in the daily press, since the ambulant intake of uncontrolled liquorice volumes is possible. A similar warning against the indiscriminate use of succus liquiritiae originates from WILDE who believes that he was able to prevent edemas, hypertonicity and electrolyte shifts in his patients by means of a salt-free diet.

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The Electrolytic Effects of Licorice Preparations in the Adrenalectomized Rat*

By WILLIAM E. HASSAN, Jr.,† JOSEPH F. PALUMBO,‡ and FRED ELMADJIAN§

Spanish licorice extract, ammoniated glycyrrhizin, monoammonium glycyrrhizinate and glycyrrhetinic acid were tested for electrolytic effects similar to those of desoxycorticosterone acetate. All the compounds tested, except glycyrrhetinic acid, produced electrolytic changes when administered orally to adrenalectomized rats. All fractions, except glycyrrhetinic acid, produced a K/Na ratio which with increased drug dosage resembles that of desoxycorticosterone acetate.

DURING the past ten years a number of reports have appeared in the literature about the use of licorice extract in the treatment of Addisonian patients. All of the reports appearing in the medical journals are the results of clinical observations and tests. To date, no controlled laboratory experiments to determine whether or not licorice extract possesses an electrolytic effect in the adrenalectomized rat have been reported.

The purpose of this investigation was to test biologically by the method of Marcus, Romanoff, and Pincus (1) the various licorice fractions available in order to ascertain by accepted laboratory methods whether or not these fractions had electrolytic activity.

HISTORY

Revers (2) in 1946 investigated the usefulness of licorice extract in the treatment of peptic ulcer. In a later report he (3) noticed that approximately 20 per cent of his patients developed edema, hypertension, or cardiac asthma or both. It was clearly evident that these symptoms could not have been due to cardiac or renal complications for it was stated that the electrocardiograms were normal, that there was no change in the urine, and that there was no diminution in serum protein content.

Borst, *et al.* (4), and Molhuysen, *et al.* (5), in 1950, demonstrated that extract of licorice exerted on human beings a biological activity which simulated that of desoxycorticosterone in so far as the electrolyte balance of sodium and potassium is concerned.

In 1951 Groen *et al.*, (6), confirmed the results obtained by Borst and Molhuysen. In an addendum to their paper it was stated that the daily administration of 3.3 Gm. of crude ammonium glycyrrhizinate kept a patient, previously maintained on 2.5 mg. desoxycorticosterone acetate daily, in sodium and potassium equilibrium.

Late in 1951 Groen, *et al.* (7), published a report that includes an observation on the effect of glycyrrhizinic acid on the electrolyte metabolism in Addison's disease. In the summary of this paper the following statement was made: "A case of Addison's disease is described in which, after the administration of desoxycorticosterone was stopped, electrolyte equilibrium was maintained by the daily administration of 3–4 Gm. of ammonium glycyrrhizinate by mouth. On withdrawal of the glycyrrhizinate administration the patient responded by sodium and chloride loss, potassium retention, and clinical signs of dehydration and hemoconcentration. Administration of crude licorice extract brought the electrolyte metabolism to normal. This observation seems to prove that the glycyrrhizinic extract of licorice exerts its influence on the electrolyte metabolism in Addison's disease."

METHOD

Male albino rats of the Wistar strain weighing between 135 and 150 Gm. were used. Each rat was bilaterally adrenalectomized under ether anesthesia and was maintained on Purina Chow diet. Physiological saline solution was used for drinking water. Cook and Elmadjian (8) used two-day and three-day adrenalectomized rats. Since they have shown that best results were obtained on these days we have also used two-day and three-day adrenalectomized rats. Food and saline were withheld for three hours prior to the experiments.

At the time of the experiments the rats were etherized to insure complete bladder emptying. The rats were then injected as indicated with aqueous solutions of the following: sodium chloride (0.9%), Spanish licorice extract, monoammonium glycyrrhizinate, ammoniated glycyrrhizin, and glycyrrhetinic acid orally, and with the oily solution of desoxycorticosterone acetate parenterally. Peanut oil was used as the vehicle for the desoxycorticosterone acetate. The remainder of the experimental procedure is similar to that described by Cook and Elmadjian (8).

The K/Na ratio for each fraction is determined by calculating the ratio for the individual pair of animals in the assay at any one dosage level. The ratios so obtained are then averaged and this figure is recorded as the K/Na ratio for the entire group

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TABLE I.—URINARY EXCRETION OF SODIUM AND POTASSIUM FOLLOWING THE INTRAPERITONEAL ADMINISTRATION OF 5-CC. NORMAL SALINE SOLUTION AND DCA SUBCUTANEOUSLY IN ADRENALECTOMIZED RATS*

Dose of DCA	No. Pairs per Assay	Urine Volume, cc.	Sodium mg./hr./100 Gm.	S. E.	Potassium, mg./hr./100 Gm.	S. E.
Control	14	6.1	1.86	±0.12	1.01	±0.03
6 μg.	8	5.1	1.49	±0.36	1.39	±0.14
12.5 μg.	8	4.6	1.06	±0.13	1.15	±0.16
25 μg.	8	3.1	0.74	±0.03	1.54	±0.18
50 μg.	15	4.6	0.96	±0.07	1.40	±0.06

* (8) Cook, M. R., and Elmadjian, F.

of assays at that particular dosage level. These ratios are recorded for each fraction in Table VI.

In this work 0.70 was set as an arbitrary ratio to indicate the lowest range of activity. In other words, any dosage level producing a ratio of less than 0.70 is to be considered inactive; and any dosage level showing a ratio of 0.70 or over is to be considered as active. In this way the activity produced by the dose of the drug can be compared with that of desoxycorticosterone acetate.

DISCUSSION

Previous workers, (1) and (8), have shown that desoxycorticosterone acetate caused a retention of urine, a decreased output of sodium ion, and an increased output of potassium ion in the urine. They have also demonstrated that the method of assay employed was sensitive to activity in doses as low as 6 μg. desoxycorticosterone acetate. Therefore it was reasonable to assume that if 5 cc. of physiological saline solution and doses of licorice extract or its fractions were administered to two-day and three-day bilaterally adrenalectomized rats, a decrease in urine and sodium output and an increase in potassium output would indicate that the licorice extract possessed an electrolytic activity. Furthermore, Groen, *et al.* (6), stated that licorice extract and crude ammonium glycyrrhizinate produced a desoxycorticosterone-like activity when administered to human beings orally, a distinct advantage over the desoxycorticosterone acetate which must be administered parenterally.

A survey of the results as presented in Tables II–VI shows that two of the three criteria that indicate activity are present: namely, the decreased urine and sodium output. The potassium ion instead of being excreted was being withheld in approximately the same proportion as the sodium ion. Therefore, since the mere comparison of these figures did not seem to indicate biological activity similar to that of the desoxycorticosterone acetate, another method of expressing the results has been employed.[1] Other workers (9) have shown that the sodium potassium ratio is more sensitive to DCA-like activity than the change in the excretion of each ion taken by itself. According to this method of ratio expression increased DCA-like activity is reflected by a decrease in the ratio. It was therefore felt that a simpler method of ratio expression was desirable in order to show an increased ratio commensurate with increased activity of the fraction administered. This was done by merely expressing the ratio as the potassium sodium ratio (K/Na).

RESULTS

Spanish Licorice Extract.[2]—The results obtained by the use of Spanish licorice extract are tabulated in Table II. A comparison of these data with that of the control animal data in Table I clearly indicates that this fraction induced a retention of urine and of sodium ion, particularly at the 500-mg. and 1,000-mg. dose levels. Excretion of the potassium ion was not increased significantly. In the high dose level of 2,000 mg. per animal marked diarrhea developed, which contaminated the urine specimens. Reference to Table VI, which shows the K/Na ratio, indicates that 500-mg., 1,000-mg., and 2,000-mg. doses of Spanish licorice extract produce a K/Na ratio far above 0.70.

Ammoniated Glycyrrhizin.—Table III contains experimental data obtained by the oral administration of ammoniated glycyrrhizin to adrenalectomized rats. Once again two of the three criteria indicative of DCA-like activity are present: namely, a significant decrease in sodium output and a tendency toward retention of urine. Reference to Table VI

[1] The calculation is described under section on *Methods*.
[2] Powdered extract; 4% moisture content.

TABLE II.—URINARY EXCRETION OF SODIUM AND POTASSIUM FOLLOWING THE ORAL ADMINISTRATION OF BOTH 5-CC. NORMAL SALINE SOLUTION AND SPANISH LICORICE EXTRACT IN ADRENALECTOMIZED RATS

Dose in mg.	No. Pairs per Assay	Urine Volume, cc.	Sodium, mg./hr./100 Gm.	S. E.	Potassium, mg./hr./100 Gm.	S. E.
90	24	2.2	1.68	±0.12	1.56	±0.09
180	15	4.6	1.66	±0.22	1.03	±0.03
270	10	4.7	1.78	±0.22	0.87	±0.06
500	8	3.3	1.34	±0.43	1.05	±0.36
1000	8	2.8	0.90	±0.12	0.95	±0.07
2000	6	4.0	1.69	±0.38	1.43	±0.41

TABLE III.—URINARY EXCRETION OF SODIUM AND POTASSIUM FOLLOWING THE ADMINISTRATION OF AMMONIATED GLYCYRRHIZIN IN 5-CC. NORMAL SALINE SOLUTION ORALLY TO ADRENALECTOMIZED RATS

Dose in mg.	No. Pairs per Assay	Urine Volume, cc.	Sodium, mg./hr./100 Gm.	S. E.	Potassium, mg./hr./100 Gm.	S. E.
50	21	3.9	1.27	±0.09	0.81	±0.06
100	14	3.7	1.42	±0.13	0.84	±0.06
250	18	4.7	1.55	±0.11	0.82	±0.04
500	14	3.7	1.24	±0.14	0.89	±0.05
750	7	4.7	1.35	±0.09	1.11	±0.05
1000	18	3.9	0.85	±0.09	0.68	±0.05

TABLE IV.—URINARY EXCRETION OF SODIUM AND POTASSIUM FOLLOWING THE ORAL ADMINISTRATION OF PURE MONOAMMONIUM GLYCYRRHIZINATE IN 5-CC. NORMAL SALINE SOLUTION TO ADRENALECTOMIZED RATS

Dose in mg.	No. Pairs per Assay	Urine Volume, cc.	Sodium, mg./hr./100 Gm.	S. E.	Potassium, mg./hr./100 Gm.	S. E.
50	21	2.9	0.95	±0.07	0.49	±0.04
75	14	2.9	1.06	±0.13	0.67	±0.23
100	14	5.0	1.13	±0.05	0.82	±0.22
150	10	4.7	1.17	±0.06	1.04	±0.02
200	18	6.3	1.39	±0.03	1.18	±0.06
250	21	3.6	1.19	±0.07	0.66	±0.04

indicates that doses of 500 mg. up to 1,000 mg. of the licorice fraction (ammoniated glycyrrhizin) produce a K/Na ratio above 0.70.

Monoammonium Glycyrrhizinate.—Table IV contains the experimental data obtained by the oral administration of graded doses of pure monoammonium glycyrrhizinate to adrenalectomized animals. Once again the urine retention and decreased sodium output properties of the compound are evident. Table VI shows that 100 mg. of this salt produced a K/Na ratio of 0.75.

It is interesting to note at this point the activity relationship of the three fractions discussed thus far: Spanish licorice extract and ammoniated glycyrrhizin started to exhibit activity at the 500-mg. dose level, whereas monoammonium glycyrrhizinate produces the same approximate comparative activity with one-fifth of the dose.

Glycyrrhetinic Acid.—Since the pure monoammonium glycyrrhizinate fraction exhibited the greatest activity, it was anticipated that the glycyrrhetinic acid, prepared by the acid hydrolysis of the monoammonium salt, would produce the ultimate in activity. Reference to Table V and to Table I for the controls, shows that the greatest urine and sodium retention is produced by a dose of 200 mg. of the acid. The potassium also has been retained to a marked degree. Reference to Table VI for the K/Na ratio shows that at this level the ratio is only 0.52, which we have already indicated is inactive. An interesting feature of this experiment is that the relative activity (according to the K/Na ratio) at the 50-mg. dose level steadily decreases with the increased dosage and that this decreased ratio is due to the potassium retention, which is far greater than the sodium retention.

TABLE V.—URINARY EXCRETION OF SODIUM AND POTASSIUM FOLLOWING THE ORAL ADMINISTRATION OF GLYCYRRHETINIC ACID IN 5-CC. NORMAL SALINE SOLUTION TO ADRENALECTOMIZED RATS

Dose in mg.	No. Pairs per Assay	Urine Volume, cc.	Sodium, mg./hr./100 cc.	S. E.	Potassium, mg./hr./100 Gm.	S. E.
50	14	4.3	1.44	±0.16	1.01	±0.07
75	7	4.4	1.54	±0.04	0.91	±0.15
100	14	4.2	1.77	±0.17	0.79	±0.01
200	12	2.8	1.08	±0.01	0.54	±0.05

TABLE VI.—POTASSIUM-SODIUM RATIO, K/Na

DCA Dose	DCA Ratio	Spanish Licorice Extract Dose[a]	Spanish Licorice Extract Ratio	Ammoniated Glycyrrhizin Dose[a]	Ammoniated Glycyrrhizin Ratio	Monoammonium Glycyrrhizinate Dose[a]	Monoammonium Glycyrrhizinate Ratio	Glycyrrhetinic Acid Dose[a]	Glycyrrhetinic Acid Ratio
Control	0.54	90	0.67	50	0.66	50	0.52	50	0.74
6 μg.	0.86	180	0.61	100	0.60	75	0.65	75	0.67
12.5 μg.	1.08	270	0.60	250	0.55	100	0.75	100	0.51
25 μg.	2.00	500	0.83	500	0.80	150	0.90	200	0.52
50 μg.	1.40	1,000	1.05	750	0.83	200	0.87		
		2,000	0.83	1,000	1.05	250	0.59		

[a] Dose in milligrams per animal.

When these dosage ranges are compared with what is reported to be effective in man, it is noted that the rat requires relatively large doses. It may be that the rat is not the most suitable animal for determining the biological activity of licorice.

SUMMARY

1. Spanish licorice extract, ammoniated glycyrrhizin, monoammonium glycyrrhizinate and glycyrrhetinic acid were tested for electrolytic activity similar to that of desoxycorticosterone acetate.

2. Spanish licorice extract, ammoniated glycyrrhizin, and monoammonium glycyrrhizinate, administered orally, exhibited an electrolytic activity when this activity is expressed as the K/Na ratio. It was observed that, first, there was an indication of quantitative decrease in sodium output and urine retention with increased drug dosage; second, there was little or no change in the potassium output.

3. Spanish licorice extract, ammoniated glycyrrhizin and monoammonium glycyrrhizinate produce a K/Na ratio which follows that of desoxycorticosterone acetate with increased dosage. Glycyrrhetinic acid does not follow the desoxycorticosterone acetate pattern because of its greater potassium retention effect.

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Scientific Section

Contribution concerning the therapeutic effect of the component substances in succus liquiritiae

By Joseph Klosa

The use of liquorice juice for various ailments was well-known in antiquity.[1] The watery and vaporized extract obtained from the ligneous root of the papilionacae Glycyrrhiza glabra L. , a shrub with meter-long offshoots and roots, named succus liquiritiae, won special interest during recent years thanks to the works of F.E. REVERS[2]. Accordingly, a therapeutic effect, or at least an improving effect is attributed to said extract in cases of stomach- and intestinal ulcers. This finding was confirmed by numerous authors[3,6]; its importance, however, was also minimized by others according to whom the the introduction of succus liquiritiae into the therapy of stomach- and intestinal ulcers does not constitute an improvement as compared to the customary treatment method[7,8]. Treatment with the commercially available succus liquiritiae does in fact cause a series of undesirable side effects, such as the formation of edemas ; REVERS[9] already mentioned these side-effects. Furthermore, HENNEMANN[10] describes, besides mentioning the above side effects, acute intoxication symptoms which can manifest themselves in various ways.

This brief survey of clinical research concerning the therapeutic value, or lack of value, of the succus liquiritiae shows that the components are undoubtedly of therapeutic value; however, it also shows that it is important to obtain a pure isolation of the single effective substances, which should be tested regarding their therapeutic effect and side effects. We accordingly endeavored to separate the principal component, i.e. the glycyrrhizin acid from the succus liquiritiae and we undertook the testing of the above (acid) in the course of animal experiments.

Succus liquiritiae represents a mixture of various substances. Its essential component is glycyrrhizin, a mixture of various salts, mainly of the calcium- and potash salts of the glycyrrhizin acid.

All evaluations of succus liquiritiae are based on a quantitative specification of the glycyrrhizin acid.[11] Additional component substances of succus liquiritiae are: glucose, saccharose, asparagine, a few amino acids, protein material and traces of volatile (essential) oils.

The following biological effects are now attributed to succus liquiritiae:

1. Spasmolytic effect[12,13];
2. DOCA-like effect, for which the glycyrrhizin acid is held responsible[15];
3. estrogen effect[16].

We were able, in an earlier work[17], to contribute experimentally to the spasmolytically effective principle. The spasmolytic effect, however, is minimal. It varies depending on the commercial product and is often not demonstrable. H. BERGER and H. HÖLLER[18] likewise report in a test paper on strong fluctuations of the musculotropic-spasmolytic effect of succus liquiritiae samples.

The side effects of succus liquiritiae are often attributed to glycyrrhizin acid; accordingly, the substance with the DOCA-like effect in succus liquiritiae is allegedly identical with glycyrrhizin acid. As evidence of the above, it is stated that GRÜEN and collaborators[19] were able to determine a DOCA-type effect with pure ammonium-glycyrrhizin. Furthermore, the glycyrrhetin acid is believed to have a considerably stronger effect on the electrolyte system as compared to (the effect of) the glycyrrhizin acid[21].

However, the above is contradicted by the findings of AHO[22], who could identify 17-ketosteroids in succus liquiritiae, which were held responsible for the edema formation occuring as a side effect of the succus-therapy. On the other hand, the therapeutic characteristic of succus liquiritiae is supposed to remain intact after the elimination of the glycyrrhizin acid, whereby no side effects regarding the electrolyte system are in evidence[23,24]. The elimination of the glycyrrhizin acid from the succus liquiritiae through precipitation with calcium chloride is proposed[27,28]. We wish to remark, however, that said precipitation process likewise eliminates further accompanying substances from the succus liquiritiae, apparently also the 17-ketosteroids mentioned by AHO[22].

As a result, extracts subjected to this type of treatment, while free from the customary side effects, also appear to be questionable regarding their value for therapy, also in view of the clinically known fact that stomach- and intenstinal ulcers are able to heal spontaneously. According to coinciding reports[29,30,31], the frequency of spontaneous healing is bteween 40 and 50%.

It was furthermore determined that ulcera can basically heal also as a result of a change of environment (hospital care) as well as through physical and mental relaxation and dieting.

SCHULZE[32] recently worked with a succus liquiritiae preparation freed from ballast-substances, which (preparation) contained concentrated glycyrrhizin acid. He was able to achieve convincing therapeutic results whereby the usual side effects did not occur. This does once more speak convincingly in favor of the therapeutic effect of glycyrrhizin.

This brief survey shows how contradictory the findigs are conecring the value, or lack of value, of succus liquiritiae application on the one hand, and on the other hand, it shows how confusing opinions are on the function of the glycyrrhizin acid in the therapeutic process. A continued investigation of the literature reveals few data specifying the preparations and their characteristics which were used for the tests. The dark brown, almost resinous ammonium-glycyrrhizin is thus widely used as glycyrrhizin acid[32]. But this product is not pure (it is commercially available) : it still contains numerous impurities. We were already able to establish the fact that numerous impurities of the succus liquiritiae are absorptively tied by Bolus alba[33], and that non-purified succus liquiritiae is more toxic; we also found that when using a purified succus liquiritiae preparation know as LAKRULCILLAN, the usual side-effects were not observed clinically[33a]. Since pure glycyrrhizin acid is hygroscopic and when left standing exposed to air, liquefies into water, we implemented our tests with the achromatic, stable potash salt of glycyrrhizin acid.

It has the following characteristic: colorless, almost odorless crystal powder with a sweet, lingering taste. The powder dissolves on the tongue into a sticky mass with pleasnt taste, with a strong expectorant effect.

Blood formula:

The potash content of the investigated preparation amounts accordingly to 4,09 - 4,36%. It represents the mono-potash salt of the glycyrrhizin acid. Using trtituration the potash salt can be easily specified.

Smp.: becomes dark at 220°C and melts while decomposing at 265 to 270°C. Soluble in warm and cold water. The watery solutions foam; easily soluble in 96% alcohol, insoluble in benzol, ether or in the customary solvents on a hydrocarbon base.

The salt was obtained according to the works of TSCHIRCH and collaborators[34], T. LADE[35], ROUSSIN[36], J. HABERMANN[37], A. VOGEL[38], P. KARRER and collaborators[39], L. RUZICKA and collaborators[40], F. BERGMANN[41], as well as W. VOSS and collaborators[42]. We improved the purification process by purifying the watery solutions of the glycyrrhizin acid with adsorption agents, obtaining preparations with this method which did not contain any impurities bound absorptively to the salts of the glycyrrhizin acid.

The use of free glycyrrhizin acid in animal tests was difficult on account of it hygroscopicity.

Toxicity: mice were used to test toxicity. Accordingly, the DL50 amounts intravenously to 412 mg/kg, itramuscularily to 695 mg/kg, subcutaneously to 697 mg/kg and perorally to 1220 mg/kg.

Chronic toxicity: administration of 60 mg/kg daily to rats during 8 months. The weight of the animals was determined every third week. Result: no abnormal weight increases. The behavior of the animals did not differ from the behavior of the control animals.

Spasmolytic effect: (tests) according to MAGNUS, using the isolated intestine of rats and guinea pigs, contraction was induced with Doryl (10^{-7}), bariumchloride (4×10^{-4}) and histamine (10^{-4}). The potash salt of the glycyrrhizin acid showed no effect; therefore it is spasmolytically ineffective.

Frog heart in situ: according to the ENGELMANN suspension method. Up to doses of 10 mg/kg of frog (weight): no effect on amplitude or frequency.

Blood pressure: rabbits and cats were subjected to examination. With a dose of 10 mg/kg no blood pressure reaction could be verified (urethane narcosis, blood pressure in the carotid, Hg-manometer). No blood pressure reaction could be detected in the cat with a dose of 50 mg/kg i.v. either; while in rabbits which received a dose of 50 mg/kg blood pressure slowly decreased to 60% of the initial value; it remained stable at this point for about 8 minutes, then slowly returned to the normal value. The period of time from the injection until the initial value was reached amounted to an average of 29 minutes.

Effect on urine excretion: total number 32 rats, of which 16 were treated and 16 served as control animals; all fasted for 16 hours. Subsequently 16 of the test animals received 5 ccm of an 1% watery solution per 100 g of body weight per os. The control animals received only water. The excreted urine volume was measured every 15 minutes during the first two hours and every 30 minutes during the 3rd hour. After 3 hours all tests were interrupted since by that time most of the water had already been excreted.

It resulted that the diuresis of the animals treated with glycyrrhizin acid began on the average 12 minutes later, as compared to the control animals. With the exception of this delayed start of the elimination, however, the course of the diuresis (in treated animals) is exactly the same as its course in untreated animals.

Effect on experimentally generated stomach ulcera: the stomach ulcera were generated with histamine[43,44], coffein and phenylchinolincarbon acid. With the last-mentioned substance, it is possible to generate stomach ulcers within 14 days. We worked with potash salt as well as with the commercially available succus liquiritiae. The test protocols will not be reproduced here and the result only shall be briefly stated: an 80% cure of the experimentally generated stomach ulcers would not be accomplished either with potash salt or with the glycyrrhizin acid.

The r sults with succus liquiritiae, however, were somewhat better, but the improvement was not sufficiently significant. The residual 20% improvement symptoms cannot be evaluated as successes; they are generally evaluated as dispersion limit instead[45].

C. OVERZIER[46] recently improved the control methods used for animal experiments. But no convincing result were obtained with this method either. We therefore believe that animal tests cannot yield any information on the factual value of remedies against stomach ulcers, i.e that the methods are deficient.

Prophylactic value: 48 rats were pre-treated with 0,4 ml of a 4% phenylchinolin acid solution, and divided into four groups:

Group A: received 0,4 of a 4% phenylchinolin acid solution daily (control group).
Group B: additional to the above, twice daily 0,5 ccm of a 0,5% glycyrrhizin acid solution per os.
Group C: additionally as for Group A, twice daily 1 ccm of the 0,5% glycyrrhizin acid solution.
Group D: twice daily an additional 2,0 ml of the 0,5% glycyrrhizin acid solution.

14,17 and 21 days after the start of the test 4 rats from each group were killed, dissected and the number of ulcers in the mucous membrane of the stomach was counted. Average value were computed for the purpose of statistical evaluation. Differences between the single groups resulted. In groups C and D a significant ulcer-prophylactic effect could be detected. It is noteworthy that the stomach wall of the animals which had been treated with glycyrrhizin acid was covered with a peculiar mucus layer which evidently acted as a shield against the ulcer-inducing effect of the substance (phenylchinolincarbon acid).

Effect on the blood count: No effect. The number of the erythrocytes as well as the hemoglobin content remained unchanged. The white blood (cell) count showed a normal reaction. At first, the number of the leucocytes decreased.but soon returned to the initial value. In the differential blood count a decrease of the lymphocytes occurred in favor of the (cells) with segmented nuclei, but the count returned to the intial values already after a few hours.

Effect on respiration: No effect on respiration was found in rats, mice and rabbits after the administration of the potash salt of the glycyrrhizin acid.

Effect on gall secretion: The test was carried out with ethylurethane (1,5 g/kg, intramuscularly), using narcotized rats with an acute choledocus fistula. The potash salt of the glycyrrhizin acid was intraveously injected. A gall secretion increase of 42% occurred; it lasted for about 30 minutes.

Estrogenic effect: Testing according to the ALLAN-DOISY method could not detect its effectiveness in castrated female rats.

Anticoagulant effect: A lengthening of the blood coagulation time occurred, corresponding to approximately 20% of the Dicumarol effect.

Clinical results: The pure potash salt of the glycyrrhizin acid was compressed into tablets of 30 and 50 mg and subjected to clinical testing. Others will report in detail on these results. In some cases, the examinations were carried out over periods longer than 16 months. A total of 10,000 tablets were currently made available for the clinics. 450 to 1000 mg were administered as daily doses. The results are as follows: elimination of pain in 80 to 90% of the cases within 3 days at the latest; recovery only 40 to 60%. No shifting of the electrolyte system of the blood; continuous normal blood formation. No occurence of edemas, headaches, numbness, sensations of oppression or development of hypertonicity; on the other hand, increased appetite and sense of well-being.

Discussion of the results: the pure potash salt of the glycyrrhizin acid from succus liquiritiae was separated in sufficient quantities, so that extensive animal tests and extensive clinical examinations became feasible (the preparation, named GLYRIN (Asal) passed the testing stage and is being introduced).

According to clinical tests the preparation showed an effect which is also known as resulting from succus liquiritiae preparations: speedy elimination of pain, healing of stomach ulcera to certain percentages. The aspect of the effect is identical here. It is surprising that pure glycyrrhizin acid has an analgesic effect. If this, however, is the case regarding pure glycyrrhizin acid, then it is questionable whether the minor spasmolytic effect of the

takes any effect at all. The following new pharmacological effects was detected: the potash salt of the glycyrrhizin acid increases gall secretion and is also capable, in a certain way, to reduce the blood pressure; furthermore, it has a weak blood-coagulating effect, which was clinically confirmed (private report) by head physican SZIRMAI in the Budapest Clinic, using high dosage rates. Stomach ulcers were not substantially affected in the course of animal tests; on the other hand, an excellent effect is demonstrable in animals tests as ulcus prophylacticum.

Therefore the potash salt of the glycrrhizin acid represents a substance with minor toxicity; in animals tests it has no effect on heart reaction or respiration; urine excretion is only slightly delayed; the blood count shows a normal reaction.

If we now consider these pharmacological and clinical test results, we must reach the conclusion that the glycyrrhizin acid cannot be held responsible for the side effects of the succus liquiritiae, while its curative value is considerable, but not of essential importance. It also becomes evident at this point why the results of various authors with a large variety of succus liquiritiae preparations are so discordant: first, the glycyrrhizin can be present in the succus liquiritiae in the form of various salt mixtures--Ca,K,Na,Mg-salts--the quantity of which can likewise vary, whereby the dosage could be insufficient; secondly,the glycyrrhizin acid could already be hydrolized into glycyrrhetin acid which does in fact cause an unfavorable shifting of the electrolyte system. Finally, other terpensteroids are responsible for the side effects. If, however better results were obtained with succus liquiritiae than with the allegedly raw glycyrrhizin acid, then it should be taken into consideration that the soluble protein materials have a favorable effect on stomach ulcers. We accordingly reach the conclusion that the curative value of succus liquiritiae evidently manifests itself jointly with various other substances, but that the glycyrrhizin acid is of decisive importance here. The disadvantage of such preparations the effective substance of which cannot be specified consists in insecurity concerning dosage rates and side effects.

We accordingly find that the glycyrrhizin acid can have a widely varied pharmacological effects. Its principal importance for the therapy of stomach- and intestinal ulcera presumably consists in the fact that it mechanically covers the stomach wall with a protective layer, which prevents further noxious chemical actions; this leads to an improved blood supply in the stomach wall. Therefore the glycyrrhizin acid is supposed to assist the healing trend . Its action stimulating the gall flow might likewise not be negligible from the viewpoint of the efficiency pattern.

The tests of J. VANDORFA[47] also indicate that the anticoagulant effect of the glycrrhizin acid is likewise involved in the favorable therapeutic effect. According to said findings, even serious ulcera could be improved with Dicumarol therapy.

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GLYCYRRHIZIN-INDUCED INHIBITION OF THE PITUITARY-ADRENAL STRESS RESPONSE*, ‡, §

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Since the desoxycorticosterone-mimetic action of glycyrrhizin has been reported, clinicians have been interested in its pharmacodynamics and possible therapeutic value. The minerosteroid-like activity of crude licorice extract was first observed in normal individuals (1, 2). Subsequently, both the active principle, glycyrrhizinic acid (3) and the crude extract have been shown capable of replacing desoxycorticosterone in treatment of patients with Addison's disease (3-8) or with total adrenalectomy (9). Glycyrrhizin-induced retention of sodium and water has been demonstrated in both intact (10) and adrenalectomized (10, 11) rats.

The action of glycyrrhizin further resembles that of desoxycorticosterone in its inhibitory effect on the pituitary-adrenal system (10, 12). Animals treated with glycyrrhizin develop adrenal cortical atrophy (12) and show less than normal reduction of adrenal ascorbic acid in response to histamine (10). In the present investigation two additional indices of pituitary-adrenal function, namely resistance to cold stress and capacity to mobilize glucose during fasting, were studied in normal and glycyrrhizin-treated animals.

Methods

Resistance of Mice to Prolonged Cold Stress.—The survival rate as a measure of resistance to prolonged stress was determined in glycyrrhizin-treated and untreated mice. Male mice, 8 to 10 gm., (10 untreated and 10 treated) were individually housed, and exposed for 8 hours to a temperature of 5°C. Glycyrrhizin-treated mice had received 0.4 per cent ammoniated glycyrrhizin in place of drinking water for 4 days prior to stress.

Glucose Mobilization of Rats Subjected to 48 Hours of Fasting.—Fasting of rats causes an initial hypoglycemia followed by a pituitary-adrenal response with mobilization of glucose (13). The concentration of glucose in blood after 48 hours of fasting was observed in 5 treated and 5 untreated male rats (145 to 150 gm.), the glycyrrhizin-treated rats having received 0.4 per cent ammoniated glycyrrhizin in place of drinking water for 1 week prior to the test.

* Ammoniated glycyrrhizin granules supplied by S. B. Penick and Co., New York.

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§ An abstract of this work appeared in *Fed. Proc.*, March, 1957, **16**, 314.

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Blood glucose levels were determined on tail vein blood at 0, 24, and 48 hours. The capacity to mobilize glucose was judged from the relative values at 24 and 48 hours.

RESULTS

Resistance to Prolonged Cold Stress.—Glycyrrhizin-treated mice died more rapidly than untreated mice during exposure to cold stress for 8 hours (Fig. 1). The median survival times for untreated and treated mice were 6.5 and

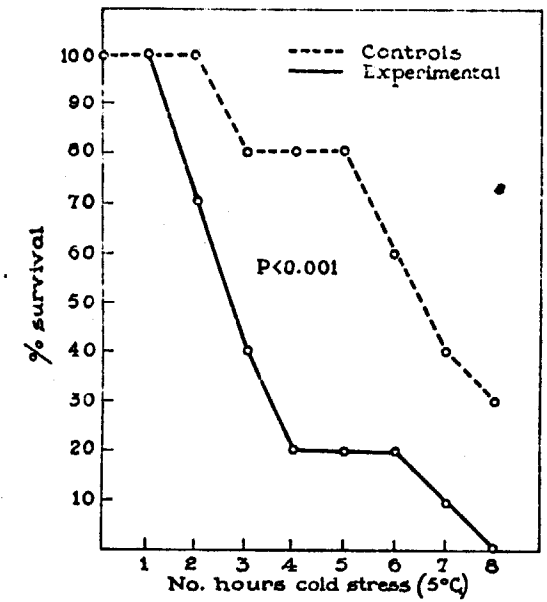

FIG. 1. Survival rate of glycyrrhizin-pretreated male mice (intact) 8 to 10 gm., subjected to cold stress (5°C.) compared with untreated controls. Experimental animals received ammoniated glycyrrhizin (0.4 per cent) *ad libitum* for a period of 4 days prior to stress. Ten animals in each group, individually housed and stressed for 8 hours. Median survival times: controls 6.5 ± 0.5 hours; experimental 2.6 ± 0.6 hours (60 per cent less than controls $P < 0.001$).

2.6 hours respectively (Fig. 1). The resistance of mice treated with glycyrrhizin was 60 per cent less ($P < 0.001$) than that of the untreated controls (Fig. 1).

Glucose Mobilization after 48 Hours of Fasting.—Glycyrrhizin decreased the ability of rats to mobilize glucose (Fig. 2). After 24 hours of fasting both groups exhibited the expected degree of hypoglycemia (Fig. 2). After 48 hours, untreated rats had mobilized sufficient glucose to bring the blood glucose level up to a value which was not significantly different from the control (Fig. 2). In contrast, the 48 hour blood glucose of glycyrrhizin-treated rats was significantly ($P < 0.01$) depressed.

DISCUSSION

Cold and fasting presumably increase adrenocortical activity through release of ACTH,[1] the degree of adrenocortical activity depending upon the quantity of hormone released from the pituitary and the responsiveness of the adrenal cortex (14). In the present study, the normal animals responded

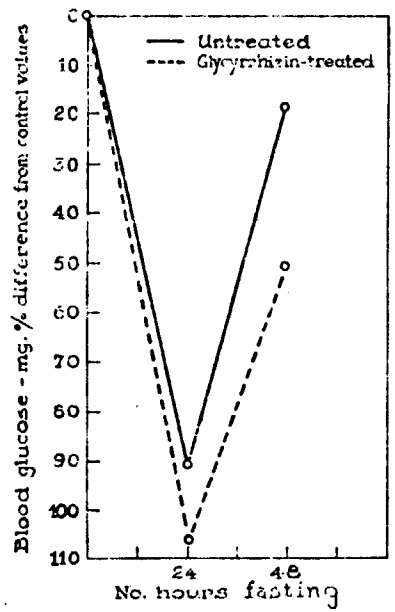

FIG. 2. Glucose mobilization response of glycyrrhizin treated and untreated intact male rats subjected to 48 hours of fasting stress. Experimental animals received ammoniated glycyrrhizin (0.4 per cent) *ad libitum* for 1 week prior to stress. 5 animals in each group. Blood glucose determined on tail vein blood at 0, 24, and 48 hours of fasting. Analysis of variance:—

1. 24 hours of fasting (hypoglycemia): (*a*) difference from control; untreated P 0.01, treated $P < 0.01$. (*b*) difference between groups; not significant.

2. 48 hours of fasting (glucose mobilization): (*a*) difference from control; untreated not significant, treated $P < 0.01$; (*b*) difference between groups; treated 22.8 per cent of the response of untreated (P 0.05).

to these stresses with increased production of corticoid, as evidenced by their resistance to the stresses and by the mobilization of glucose. Those treated with glycyrrhizin had a higher mortality and a diminished mobilization of glucose. These observations, coupled with the decrease in adrenal ascorbic acid response (10) and the atrophy of the adrenal cortex (12) observed in glycyrrhizin-treated animals, strongly suggest that glycyrrhizin depresses the output of ACTH.

[1] Adrenal corticotrophic hormone.

Desoxycorticosterone exerts a similar inhibitory effect on the pituitary-adrenal system through depression of ACTH release (15). Like glycyrrhizin it causes adrenal cortical atrophy (15, 16), diminished adrenal ascorbic acid response (15), diminished glucose mobilization and impaired resistance (16). Although the mechanism of action of glycyrrhizin is as yet unknown, its actions strongly resemble desoxycorticosterone in both its effect on the pituitary-adrenal system as well as its effect on electrolyte metabolism (1–11).

SUMMARY

Glycyrrhizin decreased the resistance of intact male mice to prolonged exposure to cold (8 hours at 5°C.) and it decreased the ability of intact male rats to mobilize glucose when subjected to 48 hours of fasting. It is suggested that glycyrrhizin, like desoxycorticosterone, depresses the output of ACTH.

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FLAVORS AND NONALCOHOLIC BEVERAGES

Quantitation of Ammonium Glycyrrhizinate by Gas-Liquid Chromatography of the Silyl Ether Ester Derivative of the Aglycone

By DAMON LARRY, MICHAEL J. FULLER, and PRINCE G. HARRILL (Division of Food Chemistry and Technology, Food and Drug Administration, Washington, D.C. 20204)

A GLC method has been developed for the quantitation of ammonium glycyrrhizinate as the aglycone, glycyrrhetic acid. The aglycone is freed from the sugar moiety in a dioxane-water mixture, using a well established acid hydrolysis procedure. The hydrolysate is extracted with chloroform, an aliquot is dried on a steam bath, and the residue is treated with pyridine - trimethylchlorosilane - N,O - bis (trimethylsilyl)acetamide reagent. The reacted product and internal standard are then chromatographed on a column of 1.5% OV-1 on Gas Chrom Q. Recoveries from both water and sodas containing caramel averaged 87%.

Ammoniated glycyrrhizin is a product prepared from the hot water extract of licorice root, *Glycyrrhiza glabra*. Sulfuric acid precipitation followed by neutralization with dilute ammonia is essentially the technique used for preparing the commercial product. One of the early methods for estimating the glycyrrhizin content of licorice root extracts, commonly called the Houseman assay (1), is the most widely used method of analysis. It is quite time-consuming and is in reality only a measure of the acid-insoluble natural extractives of the licorice root. Numerous investigators have estimated the ammonium glycyrrhizinate (AG) content of the extracted material to be from 20–90%, depending upon the number of precipitation cycles used.

The development of an acid hydrolysis procedure for stoichiometrically separating the aglycone glycyrrhetic acid (GA) from its disaccharide glycoside, glycyrrhizin, was first used and reported by Wiest (2). Van Katwijk and Huis In't Veld (3), who used an HCl hydrolysis procedure followed by a column cleanup procedure, and Cundiff (4), who added dioxane to the H_2SO_4 hydrolysis, have provided the basis for both an accurate and specific analysis.

The most comprehensive treatment of the history, chemical structure, pharmacology, and methods of analysis is found in a study by Nieman (5). It is apparent from his study and recent publications that no generally acceptable means of determining the aglycone in other than the pure extract has yet come forth. Infrared determination of glycyrrhizin in licorice extract (6) is not specific or versatile enough for other uses. This is the case also for the determination published by Cundiff (4), who used the absorption spectra of the glycyrrhetic acid-H_2SO_4-ethanol-vanillin reaction. None of the determinations studied so far, including the UV method by Van Katwijk and Huis In't Veld (3), has been suitable for quantitation to more than a very limited extent.

By far the largest user of licorice in this country is the tobacco industry as an ingredient in tobacco casing liquor (7). However, recent interest has developed for the use of ammonium glycyrrhizinate as a potentiator of sucrose sweetness. AG is reported to be a high potency natural sweetener which is 100 times sweeter than sucrose in the presence of sucrose. It is used as an intensifier of such flavors as chocolate, caramel, maple, and root beer (8). Other known uses of glycyrrhizin are as a curing agent in bacon, emulsifier in ice cream, oxidation inhibitor in chewing gum, and fat stabilizer in coating chocolate. These diverse food uses, together with the association of licorice with abnormal clinical and biochemical manifestations, emphasize the importance of developing an accurate and versatile method for determining glycyrrhizin in foods (9, 10).

The purpose of this paper is to describe a gas chromatographic method for quantitative determination of ammonium glycyrrhizinate. The aglycone obtained in a chloroform extract after acid hydrolysis in a dioxane-water mixture is

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silylated, forming an ether ester derivative suitable for gas chromatographic analysis.

METHOD

Reagent

Silylating mixture.—Mix, in order, the following (by volume): 10 parts pyridine, 2 parts trimethylchlorosilane, and 3 parts N,O-bis(trimethylsilyl) acetamide. Mix and store under nitrogen in a tightly stoppered bottle. Refrigerate when not in use. (Refrigerated reagent is stable indefinitely. A few grains of Tel-Tale silica gel indicator may be used. Reagent is suitable as long as indicator is deep blue.) Store anhydrous pyridine over 13X molecular sieve.

Equipment and Instrument Parameters

Use Barber-Colman 5000 instrument, or equivalent, with the following conditions: glass column 4′ × 4 mm id, coated with 1.5% OV-4 on 60–80 mesh Gas Chrom Q; nitrogen carrier gas 75 ml/min; sensitivity 1000; column 200–260°C at 6°/min; detector 300°C; injector 280°C.

Determination

Pipet 20 ml chloroform extract (4) into reaction container, 50 ml screw-top bottle. Add 10 ml internal standard (1 mg cholesterol/ml). Dry under stream of nitrogen on steam bath. Let cool to room temperature and add 2 ml silylating mixture. The complete silylation of the compounds requires complete solution of the residue, usually 10–15 min at 50–60°C. Stopper, cool, and use for gas chromatographic analysis.

Calculations

Determine relative response factor (RF) from chromatograms of standard $CHCl_3$ solutions of cholesterol and glycyrrhetic acid treated same as sample. Obtain peak area (PA) measurements by triangulation. RF equals peak area of glycyrrhetic acid times amount of internal standard divided by peak area of internal standard times amount of glycyrrhetic acid.

μg AG = (PA sample/PA int. std) × (μg int. std/RF) × 1.748 × 10

Results and Recommendations

The ammonium glycyrrhizinate used was prepared from technical grade ammoniated glycyrrhizin by the method of Louis and Conn (11). The identity of this pure, snow-white, crystalline product was confirmed by infrared analysis and determined to be greater than 98% pure by nonaqueous titration of the aglycone extracted after acid hydrolysis, using the method of Cundiff. Infrared analysis was also used to confirm the acid-hydrolyzed product by comparison with commercially obtained β-glycyrrhetic acid.

The chloroform-extracted hydrolysate was shown to quantitatively yield the aglycone, as presented in Tables 1 and 2 and in previous publications.

The quantitation of the aglycone by colorimetric, polarographic, and UV spectrophotometric methods is often associated with significant errors from contaminating substances not removed in the various cleanup procedures. The use of liquid-solid chromatography and gel filtration chromatography, while producing accurate results, is lengthy and in most cases has to be optimized for each different sample. Routine gas chromatography of ammonium glycyrrhizinate or its aglycone was inadequate for quantitation due to the high melting points and associated decomposition of the compounds. Investigators having similar troubles with thermally unstable biological and natural compounds were able to prepare volatile and stable silyl derivatives suitable for GLC analysis.

In this study we found bis(trimethylsilyl) acetamide, with trimethylchlorosilane as a catalyst, to be the most suitable agent for preparation

Table 1. Comparison of the results of commercial cola beverage samples, spiked with ammonium glycyrrhizinate (AG) and analyzed by the methods of Wiest (2) and Van Katwijk and Huis In't Veld (3)

Sample	AG Added, mg	Calcd GA, mg	Found, mg: Wiest	Found, mg: Van Katwijk & Huis In't Veld	Recovery, %: Wiest	Recovery, %: Van Katwijk & Huis In't Veld
1	53.5	28.8	21.0	25.6	73.0	89.0
2	80.4	43.2	30.0	38.0	69.5	88.0
3	104.1	55.5	36.8	45.0	66.4	81.0
4	50.2	27.0	18.4	20.6	68.0	76.5
5	101.3	54.5	35.0	41.0	64.2	79.5
6	None	51.5 (added)	35.8	40.6	69.5	79.0
Control[a]	35.0	20.0	19.8	20.0	99.0	100.0

[a] Water substituted for cola.

Table 2. Results of UV analysis (3) of spiked samples of cola, using the hydrolysis procedure of Cundiff (4)

Sample	AG Added, mg	Calcd GA, mg	Found, mg	Recovery, %
10	21.8	10.9	9.5	87.2
11	40.8	20.4	18.5	90.7
12	51.5	25.8	22.0	85.2
13	none	6.0 (added)	5.5	91.7
14	none	10.0 (added)	9.5	95.0
15	none	14.7 (added)	14.5	98.6

of a volatile derivative suitable for quantitative GLC. Based on the study of Klebe *et al.* (12) on the silylation of carboxylic acids, enols, and phenols as a corollary, the silylation reaction proposed here resulted in the formation of the silyl ether ester derivative of β-glycyrrhetic acid.

The BSA derivative was a quantitative conversion product and was stable for several days in a tightly stoppered bottle at room temperature. The results of the GLC analysis of samples spiked with technical and purified ammonium glycyrrhizinate and β-glycyrrhetic acid are shown in Table 3. The results indicate a very good yield with an average recovery value of 87%.

It is recommended that study be continued.

Table 3. Analysis of ammonium glycyrrhizinate (AG) and glycyrrhetic acid (GA) in water solutions and caramel-containing beverages[a]

AG Added, mg	GA Added, mg	Calcd AG, mg	Calcd GA, mg	Recovery, mg (AG or GA)	Yield, %
1000, tech. grade, in water		380		326	85.8
1000, tech. grade, in water		380		331	87.1
	250, purified, in water		249.5	206	82.6
	250, commercial, in soda		248.3	227	91.0
1000, purified, in water		950		816	85.9
1000, purified, in soda		950		892	93.9

[a] Purities: technical grade AG, 38%; purified AG, 95%; commercial GA, 99.3%; purified GA, 99.8%.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D and was accepted by the Association. See *JAOAC* **53**, 389 (1970).

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EFFECT OF GLYCYRRHETINIC ACID ON THE ADRENAL CORTEX

ANIMAL EXPERIMENTS

by

EINO LINKO and RAIMO VASAMA

(Received for publication January 11, 1958)

In animal experiments and clinical studies it has been established that glycyrrhetinic acid and its glucoside glycyrrhizinic acid exhibit properties which greatly resemble those of desoxycorticosterone. These biologically active components of licorice promote retention of sodium chloride and water in man (1,10) and have been used with advantage in substitution therapy of Addisons disease (2, 4, 7, 11). This electrolyte-regulating effect has also been demonstrated in animals (8), but the compound has not been found adequate enough to keep adrenalectomized animals alive (2,5).

Glycyrrhizinic acid decreases the excretion of 17-ketosteroids in man (9) and effects adrenal atrophy in guinea pigs (3). It has furthermore been found that rats receiving ammoniated glycyrrhizin prior to histamine stress test show a marked decrease in adrenal ascorbic acid depletion (8). These latter observations suggest that glycyrrhizinic acid may exert an inhibitory effect on the pituitaryadrenal system resembling that of corticosteroids.

The purpose of the present study was to determine the effect of glycyrrhetinic acid on the histological structure of the adrenals in the rat. Also the urinary excretion of sodium and potassium after feeding the above compound has been followed.

MATERIAL AND METHODS

The test animals were 36 male albino rats which were grouped as shown in Table 1.

TABLE 1

Group	Treatment	Duration of Treatment	Number of Animals	Initial Mean Weight g	Final Mean Weight g	Mean Change in Weight g	Mean Weight of Adrenal Gland mg 100 g of Weight
1	Control	14 days	10	221	230	9	8,6
2	600 mg Gl. acid *	10 days	4	250	266	16	8,7
3	1225 mg Gl. acid *	8 days	4	308	326	18	5,4
4	1760 mg Gl. acid *	8 days	5	180	216	36	16,5
5	Nephrectomy 1% NaCl	14 days	5		280		9,8
6	Nephrectomy 1% NaCl 2220 mg of Gl. acid.*	14 days	4	329	315	14	10,0
7	17,5 mg of Doca** per animal	7 days	4	348	358	10	7,2

* Total amount of glycyrrhetinic acid (Gl. acid) per 100 g of body weight given during treatment.

** Total amount of desoxycorticosterone acetate (Doca) given to each animal during the treatment.

The glycyrrhetinic acid was administered as an aqueous suspension through a thin polythene tube directly into the stomach. In group 6 the acid was given as a suspension in 1 per cent sodium chloride. The dose volume was 5 ml in all cases. In group 2 the amount of the acid administered was 100 mg on each of seven days and 300 mg on the eight to tenth days. In group 3 a 500-mg dose was given daily. In group 4 two 100-mg doses were given on the first seven days and two 500-mg doses on the eight day.

The animals of groups 5 and 6 were subjected to unilateral nephrectomy and then allowed to drink 1 per cent sodium chloride solution *ad libitum*. The animals of group 6 were given in addition 500 mg of glycyrrhetinic acid daily.

The animals of group 7 were given intramuscularly 2.5 mg of desoxycorticosterone acetate (»Organon») as a single daily dose.

All experimental animals were fed a standardized diet during the test period.

The weights of the animals were recorded during the test periods. In groups 3 and 7 the diurnal urine excretion was measured and the urinary sodium and potassium were determined (Table 2).

TABLE 2

Group 3

Treatment	Urine Volume ml	Urinary Sodium mg%	Urinary Potassium mg%	Body Weight g
None	18.8			
»	19.3			
»	21.3			
»	22.8			
»	24.8	750	485	
5 ml H_2O	31.8			
»	30.8			
»	33.5	382	296	
500 mg Gl.acid	31.0			308
»	28.0			
»	24.8			326
»	23.5			
»	33.8	387	399	316
»	23.5			
»	21.5			314
»	27.5	264	382	
Killed				326

Group 7

Treatment	Urine Volume ml	Urinary Sodium mg%	Urinary Potassium mg%	Body Weight g
None	20.5			
»	24.5			
»	22.0			
»	22.5			
»	23.8			
»	21.8			
»	20.3			
»	19.3	695	640	
25 mg DOCA	20.3			318
»	17.0			
»	13.8			312
»	21.3			
»	21.5	382	450	348
»	17.8			
»	17.5	384	390	348
Killed				358

The values are mean values per animal

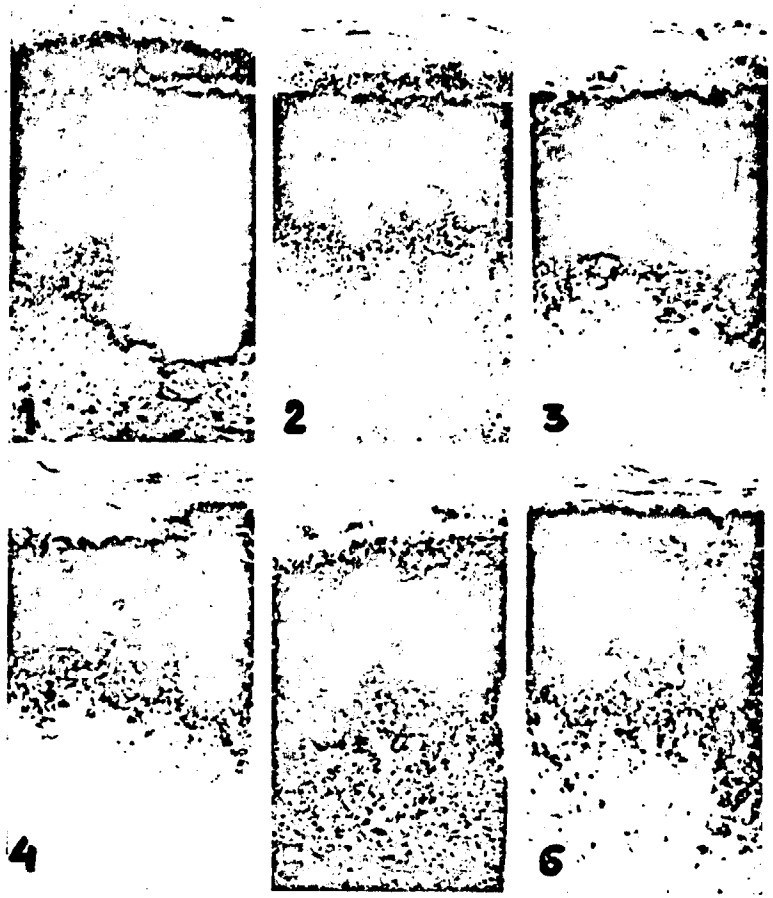
The animals were killed with a blow on the neck without narcosis on the day following the last administration of glycyrrhetinic acid. The adrenals were removed, freed carefully from adhering fat and weighed on a torsion balance. One of the adrenals was fixed in neutral 4 per cent formaldehyde, 30 μ frozen snits were made and stained with Sudan III. The other adrenal was fixed in Hellys solution during 6 hours, and the paraffin snits (7 μ) were stained with Hemalum-eosin.

HISTOLOGICAL FINDINGS

Sudan Staining. Control Group. — In the adrenal glands of the control animals the zona glomerulosa was in general fairly uniformly and strongly stained red. In some cases smaller unstained areas were seen but these were rare. The unstained transitional zone (sudanophobic zone) separated the zona glomerulosa from the fasciculata, which latter, like the glomerulata, was strongly stained red; this applied in particular to its outer layers, the inner layers being somewhat less strongly stained. In the zonareticularis only an occasional stained cell was observed (Fig. 1).

Test Groups. — Of the adrenals of the animals that were given glycyrrhetinic acid those of Group 2 differed only slightly from the controls in the staining of the lipids, although unstained areas were somewhat more often seen in the zona glomerulosa. In group 3 (Fig. 1) and Group 4 (Fig. 2) the glomerulosa was appreciably less strongly stained. Isolated lipid-containing cells were distinguished in appreciable number, but uniformly stained areas were infrequent.

The Figures 2 and 4 show selected areas where the Sudan staining is strongest in the glomerulosa. The sudanophobic zone can be distinguished less clearly in some areas but generally merges with the unstained former. In the adrenals of Groups 5 and 6 the sudanophilic material was almost completely absent from the glomerulosal zone, only a few isolated cells being stained. The sudanophobic zone could be distinguished with difficulty in only a few places (Fig. 3). In the animals of Group 7 that were given desoxycorticosterone acetate a marked depletion of sudanophilic material was observed in the glomerulosal zones of the adrenals of these animals (Fig. 5) while the amount of sudanophilic material was



Figs. 1—6. — Frozen sections 30 microns thick stained with Sudan III.

Fig. 1. — Control. The glomerulosal zone is wide and strongly stained. The sudanophobic zone is clearly evident.

Fig. 2. — Group 4. Glycyrrhetinic acid administered. The glomerulosal zone is weakly stained. The sudanophobic zone is indistinct.

Fig. 3. — Group 6. Nephrectomy + 1% NaCl + glycyrrhetinic acid. The glomerulosal zone is practically unstained and the sudanophobic zone cannot be distinguished.

Fig. 4. — Group 3. Glycyrrhetinic acid administered. Occasional stained areas seen in glomerulosal zone. Near these stained areas a narrow sudanophobic zone can be distinguished.

Fig. 5. — Group 7. Desoxycorticosterone acetate administered. Only small stained areas and isolated stained cells are seen in the glomerulosal zone. The sudanophobic zone merges with the unstained glomerulosal zone.

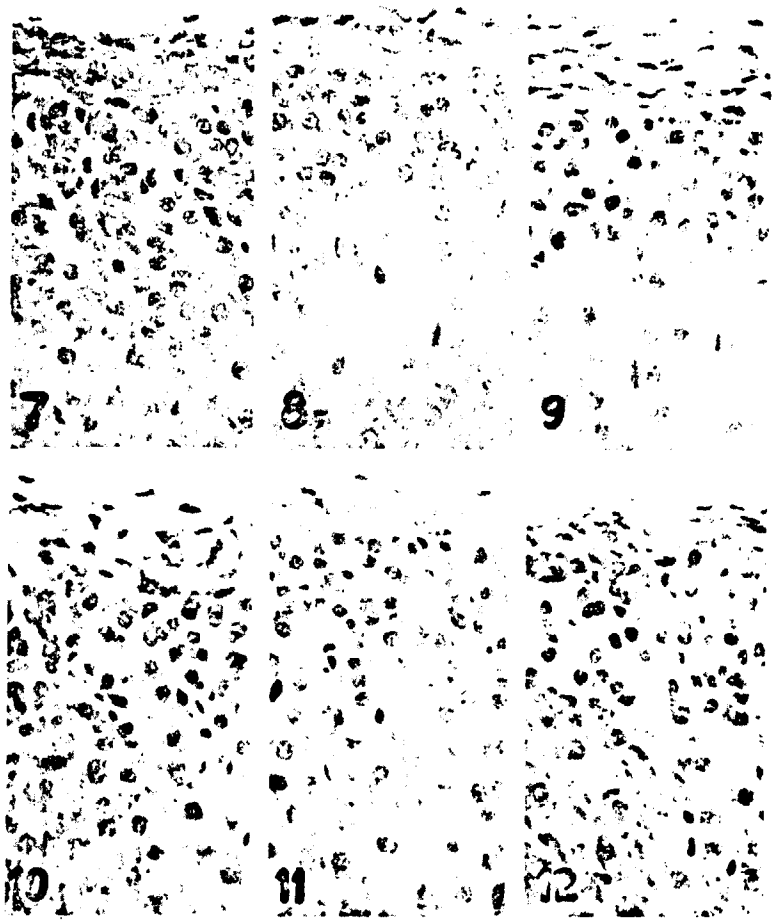
Fig. 6. — Group 7. Desoxycorticosterone acetate administered. The glomerulosal zone is wide and the most strongly stained of the zones. A narrow transitional zone can be distinguished.

so large in the adrenal of one animal that the zona glomerulosa was more strongly stained than in the controls (Fig. 6). In general the sudanophobic zone could not be distinguished in the adrenals of the first three animals and it was narrower than normal in the last case.

No appreciable differences in the staining of the fasciculata and reticularis were noted between the test-and control animals.

Hematoxylin-Eosin. Control Group. — Large numbers of vacuoles of fairly small size were seen in the plasma of the cells of the glomerulosal zone in the adrenals of the control animals. Also cells with dense plasma containing no vacuoles were observed in the zone, but they were few in number. Usually a transitional zone consisting of cuboidal cells with dense plasma separated the glomerulosa and fasciculata, but sometimes the zone was not continuous (Fig. 7). The cells of the zona fasciculata were much larger than those of the transitional zone, had large vacuoles and contained a small amount of reticulous plasma between the vacuoles. The cells of the reticularis had a fairly dense plasma and the single vacuoles found in them were small in size.

Test Groups. — The adrenals of the animals of group 2 which were given glycyrrhetinic acid did not differ morphologically from the controls, but those of groups 3 and 4 (Fig. 10 and 8) differed clearly. In the latter two groups the glomerulosal zone consisted of smaller than normal cells with, usually, dense plasma. Evidently owing to this the nuclei were seen in higher numbers than normally in this region. A transitional zone similar to that in the controls was not seen, although sometimes cells with dense plasma that were larger than those in the glomerulosal zone were located between the glomerulosa and fasciculata, but these did not form any clearly defined zone, but occurred isolated or in small groups. A large number of pyknotic cells was seen in the glomerulosal zone. The changes observed in group 5 resembled the former, while in group 6 the cells of the glomerulosal zone were very small in size and the zone as a whole seemed to be narrower. Vacuoles were only rarely seen in the cells which had dense plasma and were strongly stained by eosin. The nuclei were darker in colour than normally and pyknotic cells were found in fairly high numbers. No transitional zone was distinguished (Fig. 9). Three of the animals of group 7 who were given desoxycorticosterone acetate showed very small cells containing dense plasma in the glomerulosal zone (Fig. 11).



Figs. 7—12. — Paraffin sections. Hemalum eosin.

Fig. 7. — Control. The cells of the glomerulosal zones contain small vacuoles. A transitional zone consisting of cells with dense plasma separates the glomerulosal and fascicular zones.

Fig. 8. — Group 4. The glomerulosal cells have no vacuoles and the number of nuclei seems to have increased. The transitional zone cannot be distinguished.

Fig. 9. — Group 6. The glomerulosal cells have dense plasma and the nuclei are pyknotic. No transitional zone evident.

Fig. 10. — Group 3. The glomerulosal cells are mostly small and have dense plasma. Also a few cells with small vacuoles are visible. No transitional zone is seen.

Fig. 11. — Group 7. The glomerulosal cells are for the most part small. A few cells with vacuoles are found. The boundary between the glomerulosal and fascicular zones is indistinct. There is no transitional zone.

Fig. 12. — Group 7. The glomerulosal cells containing numerous vacuoles and small cells with dense plasma are seen only under the capsule. The few cells with dense plasma seen between the zona glomerulosa and fasciculata evidently represent a transitional zone.

Vacuoles were seen only rarely. Between the glomerulosa and fasciculata eosinophilic cells larger than the preceeding were seen. These evidently represent a transitional zone. Pyknotic cells were found in higher than normal frequency in the glomerulosal zone. In one of the cases (Fig. 12) where the sudanophilic material in the glomerulosal zone had increased, the cells of the zone stained by hemalum-eosin were generally large in size and contained numerous small vacuoles. The cells in the outer layers of the zone near the capsule had dense plasma and were eosinophilic. Small cells with dense plasma were located between the glomerulosa and fasciculata and obviously represent a transitional zone and occurred histologically in the same region where the sudanophobic zone was seen in Sudan staining.

No differences in the fasciculata and reticularis were observed between control and test groups.

DISCUSSION

Changes were noted in the histological appearance and lipid content of the glomerulosa zone in the adrenal cortex of the animals which were given glycyrrhetinic acid. A marked depletion of the sudanophilic material in the glomerulosal zone and simultaneous changes in the morphology of the cells suggested an inpaired function. In these animals the boundary between the zona glomerulosa and fasciculata had generally become more diffuse and the transitional zone that was clearly evident in the control adrenals was only seldom distinguished and even then it was indistinct. It thus seems that glycyrrhetinic acid affects also this part of the adrenal cortex. In magnitude the histological changes effected experimentally seem to be clearly dependent on the amount of glycyrrhetinic acid administered since the changes noted in Group 2 were much smaller than those in Groups 3 and 4 which were given larger doses.

In the nephrectomized animals of Groups 5 and 6 clear atrophic changes were noted in the glomerulosaq zones of the adrenal cortices as compared with those of the controls. The histological changes were somewhat more pronounced in Group 6, which suggests that glycyrrhetinic acid promoted the atrophic changes.

A comparison of the histological changes that occurred in the glomerulosal and transitional zones in the adrenals of the animals

that were given glycyrrhetinic acid (groups 2, 3, 4, and 6) with those in the adrenals of the animals which were given desoxycorticosterone (group 7) revealed that with one exception the changes were similar.

Atrophy of the glomerulosal zone of the adrenal gland did not become apparent as a decrease in the weights of the gland: on the contrary, the weights of the glands were somewhat higher in the animals that had been given glycyrrhetinic acid than in the controls (Table 1).

A clear decrease in sodium excretion was observed in Group 3 which was correlated with the weight increase of the animals and evidently also with the water retention during the test period. This result is analogous to that observed in Group 7 which was given desoxycorticosterone and also with the results relating to sodium retention in rats reported by Kraus (8). A fairly clear increase occurred in the potassium excretion in Group 3, whereas an unexpected decrease was observed in Group 7.

The histological changes resulting from the administration of glycyrrhetinic acid conform with the results of Sarason (12), Gaup and Deane (6) and Selye and Stone (13) who observed depletion of sudanophilic material and atrophic changes in the glomerulosal zone of the adrenal cortex in test animals after administration of desoxycorticosterone.

The histological findings also confirm the prevailing opinion based on results obtained using different methods by Louis and Conn (9) and Kraus (8) that glycyrrhetinic acid has an inhibitional effect on the pituitary-adrenal system.

In the present study an aqueous suspension of glycyrrhetinic acid was introduced directly through a polythene tube into the stomachs of the animals and the daily dose given varied from 200 to 1000 mg. The experimental animals were in good health during the test period, no gastrointestinal or other externally visible disturbances being noted. Despite the large doses the increases in weight of the animals, which obviously resulted from retention of sodium chloride and water, were fairly moderate. This finding does not conform with the results of earlier investigations in which 3–4 grams of mono-ammonium glycyrrhizinate have been found to induce definite clinical and endocrinological responses in humans.

The reason for this discrepancy is unknown to us. It may, however, be suspected that special characteristics of the animals used in the present experiments may have influenced the results or that the resorption of glycyrrhetinic acid differs from that of mono-ammonium glycyrrhizinate which has been employed in most of the earlier investigations. Work in progress may be expected to clarify this latter point.

SUMMARY

The effect of glycyrrhetinic acid on the histology of the adrenal gland has been studied in male albino rats. In view of previous findings the mineralocorticoid-like action of the acid has been examined by following the urinary excretion of sodium and potassium in two of the test groups.

It was established by means of morphological examination and lipid staining methods that glycyrrhetinic acid has a similar effect on the zona glomerulosa, and also to some extent on the transitional zone, as desoxycorticosterone.

No changes were found in the fasciculata and reticularis.

The histological findings confirm the previously presented opinion that glycyrrhetinic acid exerts an inhibitional action on the pituitary-adrenal system.

The excretion of sodium decreased and that of potassium increased at the same time as the body weights of the test animals increased following the administration of glycyrrhetinic acid.

Acknowledgement. — The pure glycyrrhetinic acid was prepared and placed at our disposal by Messrs. Leiras, Pharmaceutical Manufacturers, Turku, to whom we wish to express our thanks.

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PREPARATION OF GLYCYRRHIZINIC ACID, THE ELECTROLYTE-ACTIVE PRINCIPLE OF LICORICE: ITS EFFECTS UPON METABOLISM AND UPON PITUITARY-ADRENAL FUNCTION IN MAN

LAWRENCE H. LOUIS, SC.D., AND JEROME W. CONN, M.D.
ANN ARBOR, MICH.

IN 1946 Revers[1] observed that many patients suffering from peptic ulcer were benefited by ingesting a crude, watery extract of licorice. He noted, too, that about 20 per cent of the patients so treated gained weight and developed edema of the ankles. Molhuysen and associates[2] extended this work and demonstrated that licorice extract when given orally to normal persons causes retention of sodium and chloride as well as excretion of potassium. Groen and co-workers[3] and Card and associates[4] reported beneficial effects in patients with Addison's disease fed licorice extract, but upon this point there was lack of agreement.[2, 5]

Recently Borst and associates[5] have put forth the hypothesis that licorice is active only in the presence of a small amount of glucocorticoid. They observed in patients with Addison's disease that licorice was without effect but that doses of cortisone (2.5 mg.) would restore partially the electrolyte activity of the licorice. When 10 mg. of cortisone was given with licorice, the electrolyte effect of the latter was wholly restored.

The fact that the DOC-like effects of different commercial preparations of licorice are dissimilar could explain some of the apparently contradictory results. Attempts to fractionate and identify the active principle were not successful.[3-6] As far as we are aware, no chemically pure compound or compounds isolated from licorice have been studied except that reported recently by Pelser and associates.[7] They administered 6 Gm. of pure glycyrrhizinic acid (4 Gm. on the first day and 2 Gm. of the pure substance plus 2 Gm. of a crude preparation on the second day) to a patient with Addison's disease and observed significant retention of sodium and chloride.

The purpose of the present communication is to outline a simple method for the isolation of pure ammonium glycyrrhizinate in large yield from licorice extract and to report upon the metabolic and physiologic effects of this compound in man. The subjects of this investigation consisted of seven normal

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persons, two patients with congenital adrenal hyperplasia, and one patient with Cushing's syndrome due to bilateral adrenal hyperplasia (demonstrated at subsequent operation).

METHODS

1. *Preparation of Ammonium Glycyrrhizinate.*—The procedure of isolation of ammonium glycyrrhizinate from licorice is a modification of the method of Bergmann.[5] To 1.5 L. of hot glacial acetic acid (60° to 70° C.), 400 Gm. of ammoniated glycyrrhizin* is added slowly, as the mixture is vigorously stirred. After it has been stirred for about one hour at 60° to 70° C., the solution is allowed to stand at room temperature for a day or two. The mixture is filtered through a large Buchner funnel and washed with cold glacial acetic acid and finally with anhydrous ether.

The dark amorphous precipitate is placed in an evaporating dish and allowed to dry in a vacuum desiccator. After the material is somewhat dried, it is dissolved in 600 ml. of hot 80 per cent ethyl alcohol and heated in a steam bath for fifteen to twenty minutes. The solution is then placed in the refrigerator overnight for crystallization.

The semicrystalline solid is filtered through a Buchner funnel and recrystallized four or five times in 80 per cent ethyl alcohol. The brown color is removed by boiling the solution with norit several times during the purification process. The yield is 20 to 22 Gm. From the alcoholic filtrate, an additional 10 to 15 Gm. of the pure substance is obtained. The product is a snow-white crystalline substance and has the typical licorice taste. The formula and analysis† of the compound are given in Fig. 1.

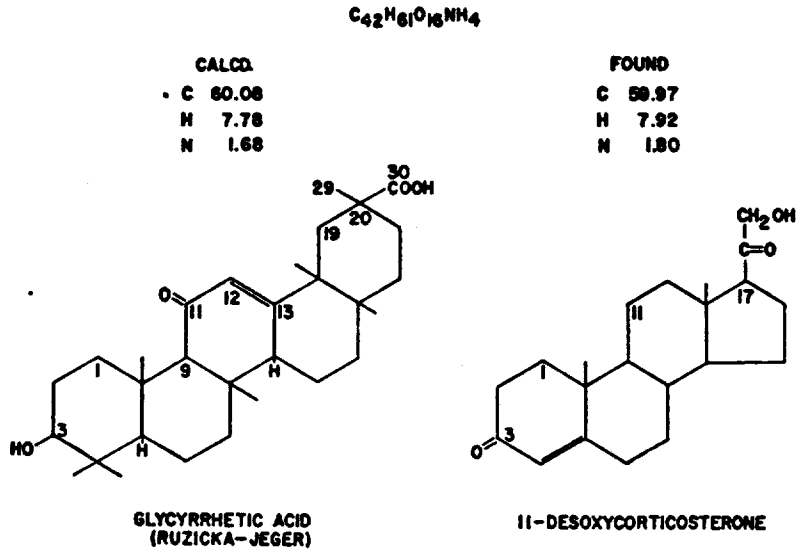


Fig. 1.—Analysis of ammonium glycyrrhizinate.

2. *Experimental Subjects and Procedure.*—Three men and four women constituted the group of normal subjects. They, as well as the patients (two girls with congenital adrenal hyperplasia and one woman with Cushing's syndrome), ingested a constant diet of average composition. In accordance with the rule of this laboratory, the subjects ingested the diet for at least three days before control data were obtained.

After a suitable control period had been obtained, ammonium glycyrrhizinate was administrated orally four times daily (8 A.M., 12 M., 6 P.M., and 11 P.M.). The amounts

*The Polychemical Company, New York, N. Y.
†Schwarzkoph Microanalytical Laboratory, Long Island, N. Y.

administered and the duration of administration of this active compound are indicated in the various figures. Fasting blood sugar, hematocrit, total circulating eosinophil cells, blood pressure, and body weight were determined daily. Glucose tolerance tests were performed before, during, and following administration of the compound. Serum sodium, potassium, and chloride were determined at frequent intervals. Total daily urinary excretion of sodium, potassium, chloride, glucose, uric acid, nitrogen, and 17-ketosteroids was determined. A series of samples of thermal sweat were collected from two of the normal subjects. These were analyzed for their electrolyte composition.

The chemical methods used in the various determinations have been described previously.[9, 10] Sodium and potassium were determined in a Beckman flame photometer.

RESULTS

1. *Effects of Ammonium Glycyrrhizinate Upon Organic Metabolism.*—In none of the ten subjects studied was any significant effect observed upon protein or carbohydrate metabolism; nor was any effect demonstrated upon the renal excretion of uric acid and creatinine or upon the level of circulating eosinophils.

2. *Effect of Ammonium Glycyrrhizinate Upon Electrolyte Metabolism.*—Here very significant effects were demonstrable on all normal subjects although considerable variation in the intensity of effects was observed from subject to subject.

The data on four of the normal subjects (typical of all) are presented in Figs. 2 to 5. The most intense effect is upon retention of sodium and chloride. In contrast with the findings of the Dutch workers,[2] we observed only a small excretion of extra potassium. Upon withdrawal of ammonium glycyrrhizinate, there occurred slight retention of potassium and great loss of sodium and chloride. Body weight did not return to original levels for five to seven days following cessation of administration of the compound.

The patient with Cushing's syndrome retained sodium and chloride and lost potassium when given the compound, as did the normal subjects. It is of considerable interest that no effect of ammonium glycyrrhizinate upon electrolyte metabolism was demonstrable in either of the cases of congenital adrenal hyperplasia. Yet in all three situations (normal subjects, Cushing's syndrome, and congenital adrenal hyperplasia), the compound appears to depress endogenous ACTH (see following).

3. *Effect of Ammonium Glycyrrhizinate Upon Excretion of 17-Ketosteroids.*—In these experiments it was demonstrated repeatedly that 17-ketosteroid excretion could be depressed by administration of the compound. This phenomenon was observed most clearly in normal subjects with high levels of 17-ketosteroid excretion and in the patients with congenital adrenal hyperplasia.

The effect of ammonium glycyrrhizinate on the renal excretion of 17-ketosteroids on three normal subjects, the two patients with adrenogenital syndrome, and the case of Cushing's syndrome is presented in Figs. 6 to 9.

Fig. 6 demonstrates that administration of the compound produces a significant decrease of urinary 17-ketosteroids in normal persons. Clearly, this is not the degree of depression which can be produced by administration of some adrenocortical compounds. Of the remaining four normal subjects

(Figs. 2 to 5), three showed a decrease of 17-ketosteroid excretion while one showed no change (Fig. 5). In the latter case control values were below 5 mg. per day.

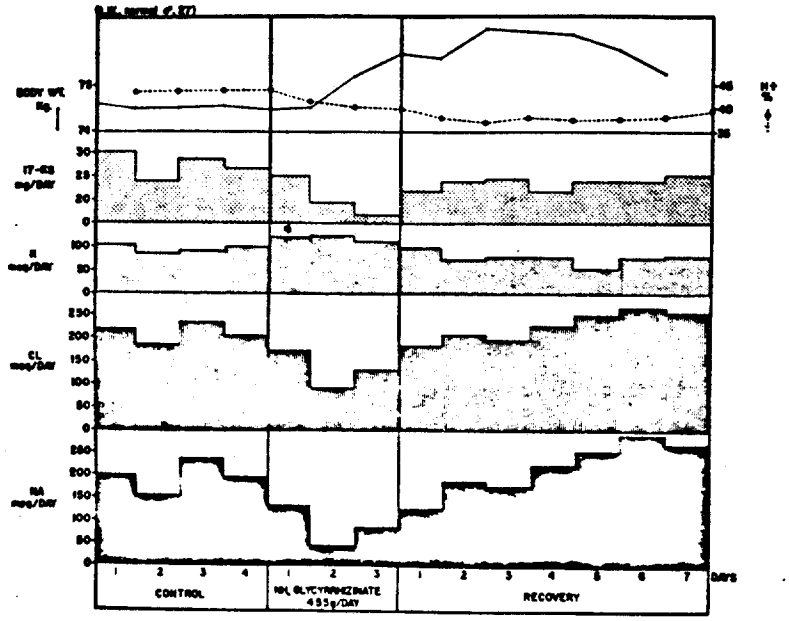


Fig. 2.

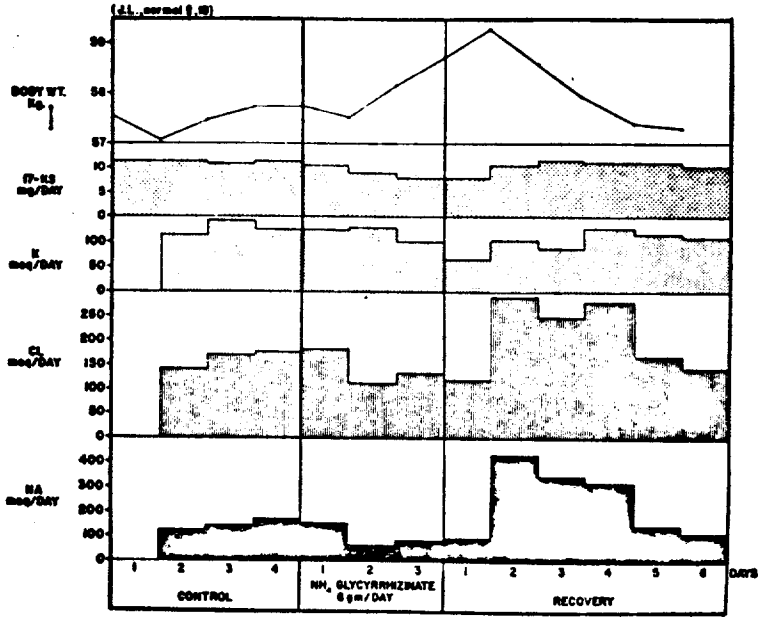


Fig. 3.

Figs. 2 and 3.—Effect of ammonium glycyrrhizinate on the renal excretion [illegible] potassium, and 17-ketosteroids.

In the case of congenital adrenal hyperplasia with very high levels of 17-ketosteroids (Fig. 7), the depressing effect of the compound is much more clearly demonstrated. It is equally clear that 120 mg. of cortisone (30 mg. orally every six hours) is considerably more effective in this respect.

The second case of congenital adrenal hyperplasia (Fig. 8) shows the effect on 17-ketosteroid excretion of increasing doses of ammonium glycyrrhizinate. Again the reduction of urinary 17-ketosteroids is evident.

In the case of Cushing's syndrome with normal base-line values for 17-ketosteroids (Fig. 9), administration of the compound for seven days (left side of the figure) and again for thirty-one days (right side of the figure) reduced 17-ketosteroid excretion only mildly, but definitely.

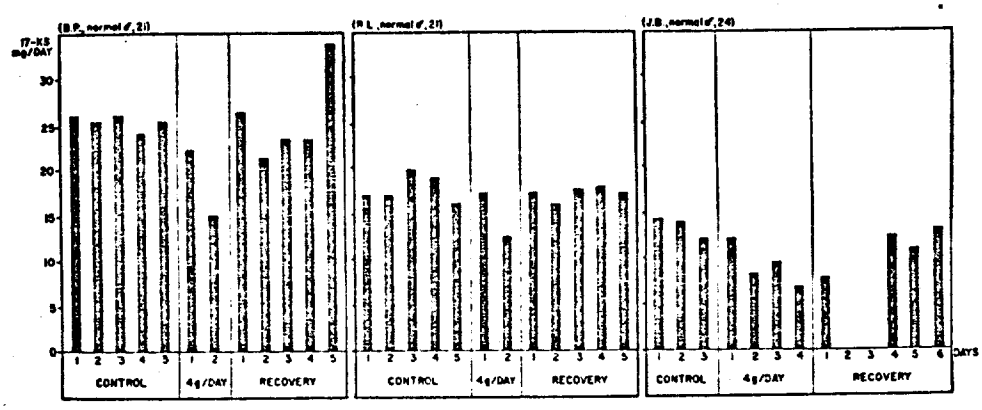


Fig. 6.

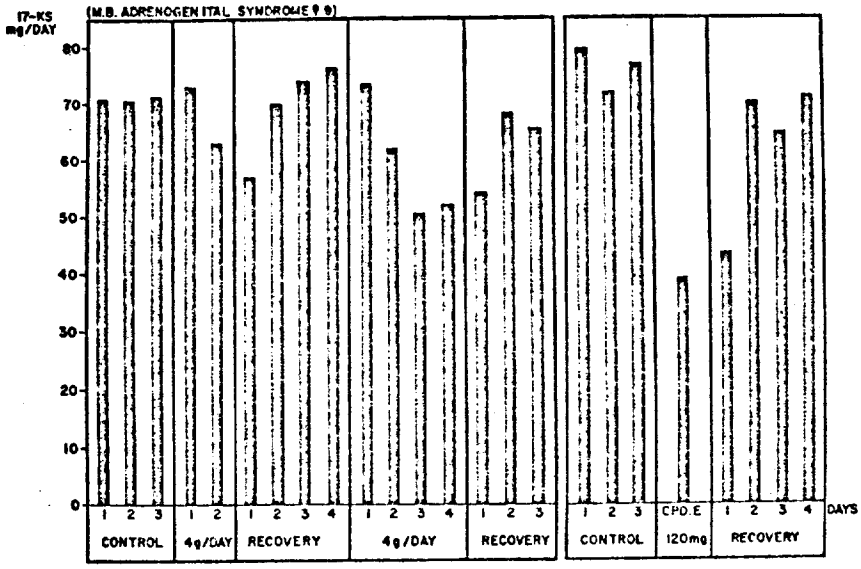


Fig. 7.

Figs. 6 and 7.—Effect of ammonium glycyrrhizinate on the renal excretion of 17-[illegible]

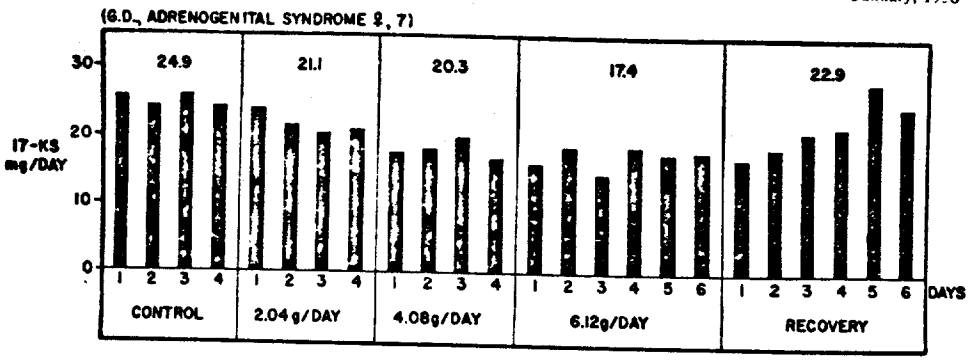


Fig. 8.

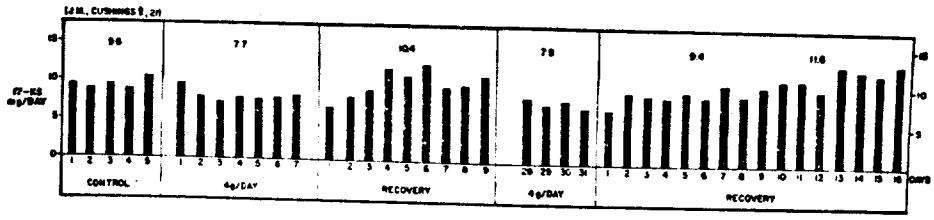
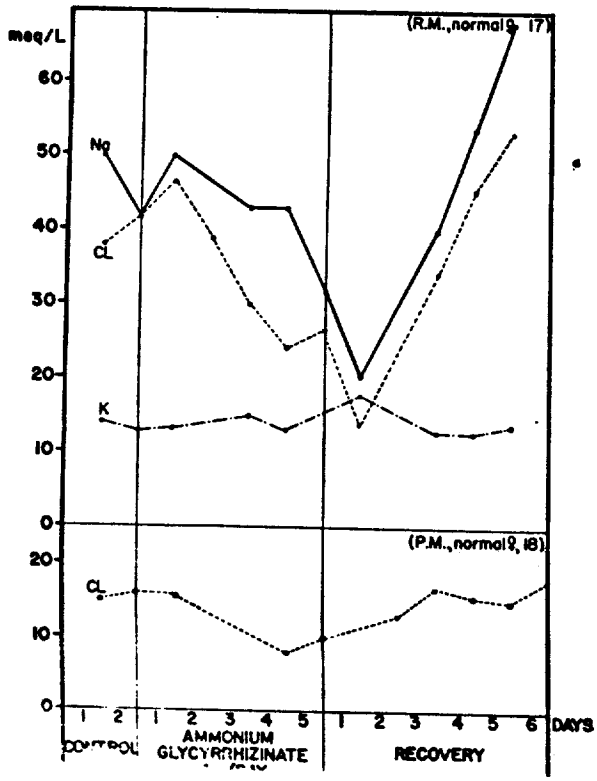


Fig. 9.

Figs. 8 and 9.—Effect of ammonium glycyrrhizinate on the renal excretion of 17-ketosteroids.



4. *Effect of Ammonium Glycyrrhizinate Upon Electrolyte Composition of Thermal Sweat.*—Since those adrenal steroids which exhibit intense sodium-retaining activity influence significantly the electrolyte composition of thermal sweat,[10-14] the effect of ammonium glycyrrhizinate in this respect was explored. Fig. 10 demonstrates that the results are those which would be expected of a potent sodium-retaining adrenal cortical steroid. On Subject P. M., only the chloride concentration of sweat was determined. The decrease in concentration of sweat chloride was much less than in Subject R. M., but the base-line values were already at the lower limit of normal.

5. *Effect of Ammonium Glycyrrhizinate Upon Urinary Excretion of Melanocyte-Stimulating Hormone (MSH).*[15, 16]*—A patient (alopecia areata) with abnormally high levels of urinary MSH was given ammonium glycyrrhizinate, 4 Gm. per day orally for a period of ten days. By the fourth day urinary MSH had fallen to normal levels. Within one day following cessation of administration of the compound, urinary MSH was back to its original high value. With respect to this activity (inhibition of pituitary release of MSH), 4 Gm. per day of this compound was found to be roughly equivalent to 5 mg. per day of 9-alpha fluorohydrocortisone, 20 mg. per day of hydrocortisone, and 37.5 mg. per day of cortisone. All these tests were carried out in the same patient.

CONCLUSIONS

Ammonium glycyrrhizinate, the sodium-retaining principle of licorice, has the following metabolic and physiologic effects when administered orally to man:

1. It induces great retention of sodium, chloride, and water.
2. It produces a mild increase of urinary potassium.
3. It inhibits mildly endogenous production of ACTH as indicated by consistent decreases in the excretion of 17-ketosteroids.
4. It decreases the concentrations of sodium and of chloride in thermal sweat.
5. It is capable of inhibiting pituitary release of MSH (melanocyte-stimulating hormone), the effect of 4 Gm. per day orally being roughly equivalent to 37.5 mg. of cortisone, 20 mg. of hydrocortisone, and 5 mg. of 9-alpha fluorohydrocortisone given orally per day, respectively.
6. It provokes no demonstrable effects upon organic metabolism (protein, carbohydrate, uric acid, creatinine, or circulating eosinophils).

The various demonstrable effects are highly suggestive that ammonium glycyrrhizinate is acting within the body in a way very similar to an adrenal cortical steroid; that its peripheral effects are exclusively upon electrolyte and water metabolism but that it is, nevertheless, capable of depressing endogenous production of both ACTH and MSH. Whether these activities are inherent in the compound as administered or result from metabolic transformations of the compound within the body must await more detailed studies. The

*We are greatly indebted to Dr. A. B. Lerner, University of Oregon Medical School.

possibility of an hepatic metabolic transformation seems likely since we have failed to elicit any effects when as much as 90 mg. per day was administered intramuscularly. This result, however, may prove to be simply a matter of dosage.

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Comptes Rendus (Biologie)
157:12, 2255-2257, 1963

Hypertensive action of various compounds prepared from licorice in normal rats.

by

J. Macabiès, A. Barbe, A. Orsetti and P. Cristol.

It has been shown (1) that ammoniated glycyrrhin administered per os can give rise to reversible and reproducible arterial hypertension in the normal rat.

The hypertensive action of this salt has been confirmed when administered parenterally and also the possible action of various related compounds.

We have studied ammonium glycyrrhizin intraperitoneally and subcutaneously, tripotassium glycyrrhizin intraperitoneally, "deglycyrrhizinated extracts" of licorice orally and the α and β isomers of glycyrrhetinic acid subcutaneously.

All our experiments were made on male Wistar rats divided into lots of 5 to 10 animals. Treatment was continued for 10 to 25 days according to the product used. Blood pressure was measured every other day with a small-animal recording oscillometer (type FC 12 Phsyiocontrol) on unanesthetized animals.

The dosages of substances administered were as follows: Intraperitoneal ammoniated glycyrrhizin: 150 mg/kg and 300 mg/kg; subcutaneous ammoniated glycyrrhizin: 300 mg/kg; intraperitoneal tripotassium glycyrrhizin: 150 mg/kg and 300 mg/kg; "deglycyrrhinated" licorice extract per os: 800 mg/kg; α and β glycyrrhetinic

acid: 300 mg/kg.

Fig. 1,2 and 3 show the variations in blood pressure during and after cessation of treatment.

Study of these curves shows that all the salts of glycyrrhinic acid have a hypertensive action and that discontinuation of treatment results in a return to normal of arterial blood pressure. The isomers of glycyrrhetinic acid also have a strong hypertensive action, but the time of action is shorter. This free acid appears to be more toxic than the salts, particularly the β isomer.

The so-called "deglycyrrhizinated" extracts which contain 3 to 4% glycyrrhizin, while the total extract contains 20 to 25%, only have a very weak hypertensive action.

Conclusion

Glycyrrhetinic acid is a compound with a hypertensive action, present in conjugated form in all products prepared from licorice used by us, which themselves proved to have an effect on arterial blood pressure.

Figures.

1. 1. Days. 2. Arterial blood pressure in mm Hg. 3. Subcutaneous. 4. Intraperitoneal. 5. Ammoniated glycyrrhizin. 6. treatment.

2. 1. Days. 2. Arterial blood pressure in mm Hg. 3. Ammoniated glycyrrhizin. 4. Ammonium chloride. 5. Tripotassium glycyrrhizin. 6. Potassium chloride. 7. Treatment. 8. Intraperitoneal.

3. 1. Days. 2. Arterial blood pressure in mm Hg. 3. Glycyrrhetinic acid. 4. Propylene glycol control. 5. Treatment. 6. Intraperitoneal.

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See footnote reference at bottom of p. 2255.

Physiological Laboratory, Director: M.L. Helon, Medical Faculty, Montpellier.

Hypertensive Action of Glycyrrhizin in Rat

by

Jean Macabiès, Annie Barbe and Pierre Cristol.

Among the various components of liquorice sugar, glycyrrhyzin appears to possess hypertensive properties. Several authors have reported such an effect, both in man (1,2) and in animals (3). However, in all cases these had been rendered delicate by some pathological (chronic alcoholism) or experimental condition (unilaterally nephrectomized rats).

An attempt was made to elicit hypertension in dogs and rats by daily ingestion of glycyrrhizin, but this failed (4).

In order to determine whether a liver disease might not make it possible to detect the hypertensive action of this product, experimentation was resumed under the following conditions: 30 male Wistar rats (initial weight 100 g to 160 g) were divided into 3 lots. The animals of lot I (6 rats) received a standard diet (Extralabo M 25). The animals of lot II (12 rats) received a low-protein diet: Protein 12.3%, lipids 15.7%, carbohydrate 72.0%. The animals of lot III (12 rats) received the same low-protein diet supplemented with a beverage with a strong alcoholic content: 96° ethanol 15 ml, water q.s.p. 100 ml.

The animals were weighed regularly twice every month throughout the duration of experimentation. Their blood pressure was measured usually on the days of weighing, by means of a

recording oscillometer for small animals (Type FC 12 Physiocontrol) during brief ether anesthesia.

After a 10-day period of observation our experiment continued for four successive periods: 1) for 70 consecutive days all the animals received 1 ml of a 16% glycyrrhizin solution daily through a stomach tube, that is 160 mg glycyrrhizin per rat per day; 2) during the next 50 days nothing was changed in the diet, but no glycyrrhizin was administered; 3) after this lapse of time, that is 130 days after the beginning of the experiment, the animals of lots II and III received the same diet as those of lot I (balanced and without alcohol); from the 130th to the 165th day (for 35 days) glycyrrhizin was again administered daily; 4) from the 165th to the 185th day (20 days) glycyrrhizin was once more discontinued.

Nothing of note was observed with regard to comparative weight increases in the various lots.

The figure shows 3 curves representing the mean values (arithmetic means) of the blood pressure of the animals from lots I, II and III during the 185 days of experimentation.

All the animals without exception responded to the ingestion of glycyrrhizin with a significant and sometimes considerable increase (up to 195 mm Hg) in blood pressure. This hypertension became apparent within a few days, regardless of the diet. The hypertensive plateau thus obtained on the average was 25% above the initial value. Even though the diets were maintained, as soon as the administration of

glycyrrhizin was discontinued, the blood pressure of all the animals dropped towards the initial level, the development of the 3 curves being entirely comparable. A further administration of glycyrrhizin makes it possible to reproduce the hypertensive phenomenon faithfully, a phenomenon which immediately yields to the cessation of treatment.

In high doses glycyrrhizin thus has a hypertensive effect on the rat; hypertension reaches its maximum within 15 to 20 days and persists so long as treatment is continued. It disappears as soon as the administration of the product ceases.

In the dose used there is no need to sensitize the animal with a hepatic disease of nutritional origin. The effect appears just as readily and with the same intensity in normal animals and pre-cirrhotic animals.

The hypertensive phase does not coincide with an excessive weight gain and the return to normal blood pressure is not accompanied by increased diuresis or loss of weight. It would thus seem difficult to explain the arterial hypertension by water retention.

Experimentation on dogs is currently in progress to clarify the mechanism of the hypertensive action of glycyrrhizin.

Summary

The daily per os administration of 160 mg glycyrrhizin to normal rats gives rise to a constant, lasting and reversible elevation in blood pressure. This elevation cannot be explained by water retention.

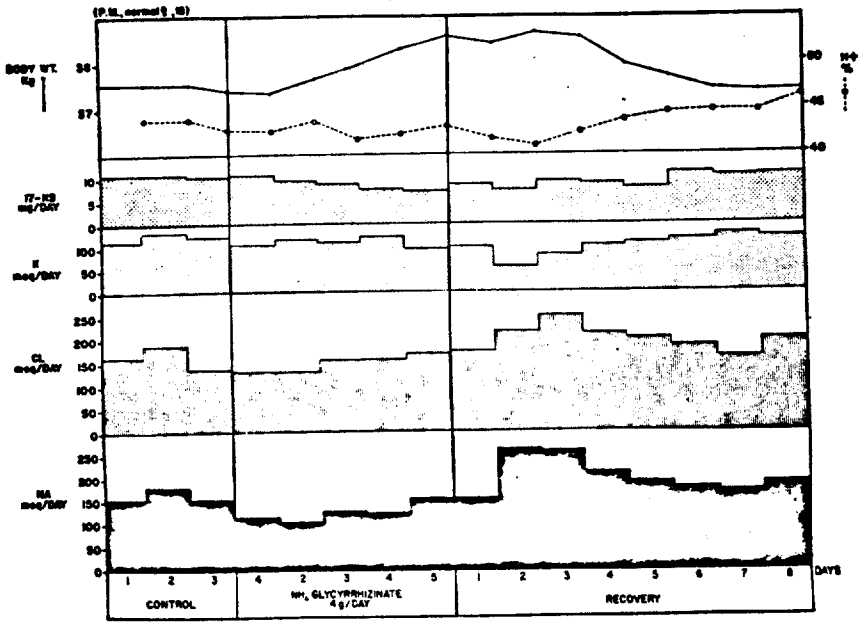

Fig. 1.

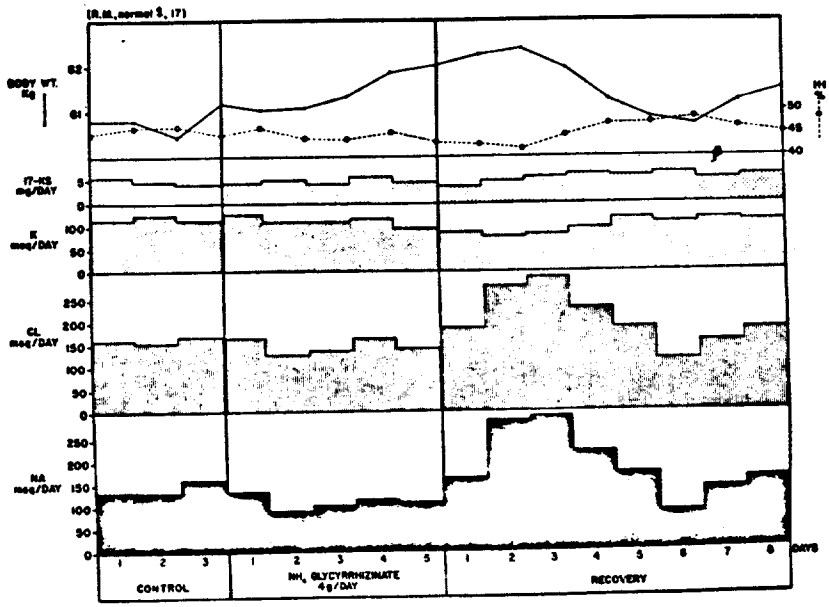

Fig. 5.

Figs. 4 and 5.—Effect of ammonium glycyrrhizinate on the renal excretion of sodium, chloride, potassium, and 17-ketosteroids.

Figure.

Abscissa: Days. Ordinate: Blood pressure in mm Hg.

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See references in footnotes, p. 1665.

A LIQUORICE EXTRACT WITH DEOXYCORTONE-LIKE ACTION *

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DURING the war years Revers (1946) noticed that many patients suffering from peptic ulcer improved after taking a proprietary preparation purchased from a local pharmacist in a small Netherlands town. This preparation was found to consist of a mixture of succus liquiritiæ, fructus anisi, and ferrum reductum. Supposing that the therapeutic effect was due to the liquorice, he treated a series of gastric-ulcer patients with a paste consisting of 2 parts powdered succus liquiritiæ and 1 part water. The patients had to take one teaspoonful three times a day, on an empty stomach. No restrictions in diet or activity were imposed. The majority of the patients became symptom-free within a short time; X-ray examination revealed that sometimes a deep ulcer of the lesser curvature disappeared within three weeks.

In 1945 succus was no longer available. In 1947 the same favourable results could be obtained, but this time serious side-effects were observed, which had not been seen before. Approximately 1 in 5 patients treated with the paste developed œdema, usually noticed first in the face and later in other parts of the body. Many patients complained of headache, and some noticed shortness of breath on exertion and pain in the upper abdomen (Revers 1948).

On reducing the dosage the symptoms usually subsided, but in some patients they remained so severe that the treatment had to be discontinued. Patients who had once responded with œdema developed it again when the drug was given a second time. But Dr. Revers did not regard the reaction as allergic; for there was no rise in the eosinophil-count and anti-histamine drugs were ineffective. He recalled having previously seen a case in which œdema disappeared when the patient stopped eating sweets containing liquorice and returned as soon as she began to eat them again. Another case of the same kind was reported by Dr. G. van den Berghe at a meeting in Amsterdam on Feb. 25, 1950. Dr. H. Pannekoek mentioned a patient who had never had symptoms of cardiac failure but developed severe cardiac asthma after taking liquorice for his stomach complaints, and several similar cases have since been noted by Belgian and Dutch doctors.

PRESENT INVESTIGATION

Succus liquiritiæ is a dried watery extract of the roots of *Glycyrrhiza glabra*. It contains 15% protein substance, 5% glycyrrhizin, 40% "extractable" substances, sugar, and 5% insoluble substance. It is generally used by pharmacists to make pills and to mask unpleasant tastes. (The quantities used for this purpose are, we have found, not much less than those known to provoke cardiac asthma in susceptible elderly people.)

After Revers reported his findings, investigations in the pharmacological institute of the University of Utrecht demonstrated that the drug had a powerful spasmolytic action and also prevented experimental production of gastric ulcers in rats and guineapigs. Glycyrrhizin was shown not to be the active principle. Administration of succus liquiritiæ to several species of animals failed to produce œdema, nor could any influence on water and sodium-chloride metabolism be demonstrated (Nelemans-Stamperius 1949).

Obviously the problem of the œdema had to be studied clinically. The possible causes of generalised œdema are:

1. Reduction of serum-albumin to less than 60% of normal.
2. Increased permeability of the capillary walls, reducing the effect of the colloid osmotic pressure of the serum-proteins.
3. Disturbance of cardiac function leading to heart-failure.
4. Primary retention of water and sodium chloride by the kidney, due to (*a*) damage to the kidney leading to a severe impairment of renal function, or (*b*) hyperfunction of the tubules reabsorbing water and sodium chloride excessively.

As possibilities 1 and 4*a* were soon ruled out, we had to decide which of the three other mechanisms was responsible for the fluid retention. They can be distinguished by the scheme shown in the following table, and in order to apply it we had to collect data on the changes in hæmoglobin, total proteins, venous pressure, blood-pressure, and pulse-pressure.

SCHEME FOR ASCERTAINING CAUSE OF ŒDEMA

	Hæmoglobin and total proteins	Venous pressure	Blood-pressure (systolic)	Pulse-pressure
Increased capillary permeability ..	Increased	Decreased	Decreased	Decreased
Primary heart-failure	Decreased; sometimes secondarily increased	Increased	Decreased or unchanged	Decreased or unchanged
Primary renal retention of water and sodium chloride	Decreased	Increased	Increased	Increased

During the past eighteen months we have given succus liquiritiæ to seven patients in our department who, in spite of long-continued dieting and bed rest, had persistent symptoms and X-ray evidence of gastric ulcer, and to three other patients. So as to get reliable data on the electrolyte balance and on the time-relations between the use of the drug and retention of water and sodium chloride, we gave eight of the ten patients a strictly standardised diet, consisting of exactly the same amounts of milk, biscuits, and butter, at three-hourly intervals by day and by night, just meeting their calorie requirements. Before receiving food at exactly midnight, 3 A.M., 6 A.M., &c., the patients passed urine. All 8 specimens were measured and examined for chloride and for urea plus ammonia. All the patients showed the excretory rhythm described elsewhere (Borst and de Vries 1950).

With each feed, the patients received supplements of constant amounts of sodium chloride and sodium bicarbonate.

Having determined the pattern of excretion for several days, we started administering the drug, giving 20–45 g. of succus liquiritiæ, also divided into 8 equal parts daily. All the patients responded in the same way, though there were striking quantitative differences. As a rule the third specimen of urine was slightly, and the fourth definitely, smaller in amount than the corresponding specimens of previous days. The chloride output decreased accordingly. The excretion of urea plus ammonia did not decrease, nor did the urea clearance. No albumin or abnormality of urinary sediment was found in any of the cases. The blood-hæmoglobin level

* Based on a lecture read before a combined meeting of the Association of Belgian Physicians and the Association of Dutch Physicians. The work was aided by a grant from the Dutch Foundation for Scientific Investigations.

fell appreciably, while the venous pressure, pulse-pressure, and systolic pressure increased significantly. After a few days, intake and output were again in balance, but the patient had gained several kilogrammes in weight.

None of our nine intensively studied patients developed clinical œdema, but some of the patients treated with liquorice for gastric ulcer, who were attending the outpatient department, had definite œdema of the legs.

Obviously the main effect of the drug was stimulation of the renal tubules to an excessive reabsorption of water and chlorides, and probably also of sodium. Results of similar magnitude and nature were obtained in a patient who received equivalent amounts of a product purified by dissolving the alcohol-soluble part of succus liquiritiæ in water and producing a precipitate by acidification. This precipitate was redissolved in water and ammonia and re-precipitation resulted in a substance which consisted mainly of glycyrrhizin.

In another patient ammonium glycyrrhizate, purchased from a wholesale dealer, produced a marked retention of extracellular fluid equal to that caused by the fraction precipitated from a solution of succus liquiritiæ by strong hydrochloric acid. On the other hand the neutralised filtrate when given in equivalent amounts to the same

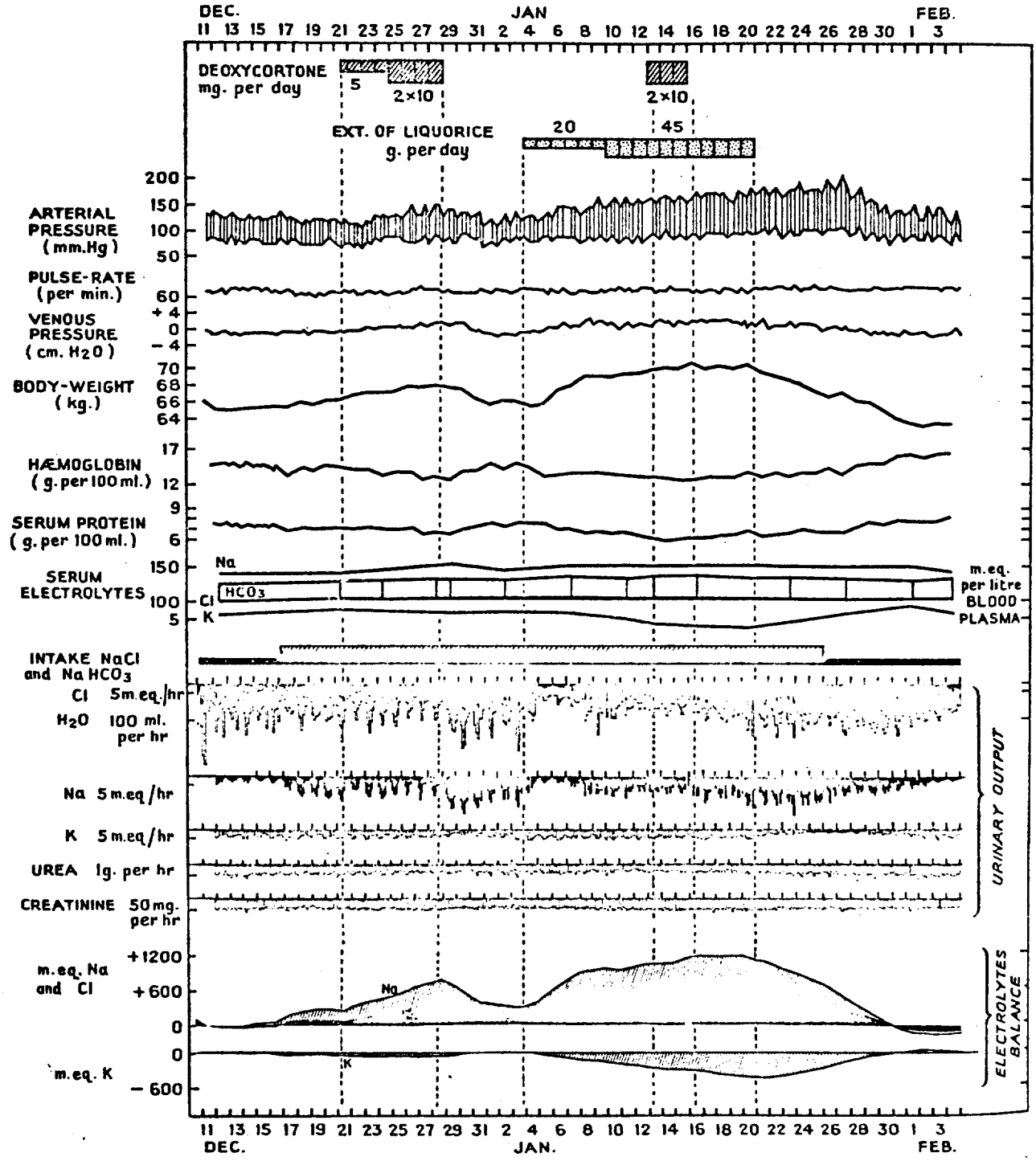

Fig. 1—Three-hourly output of water, sodium, potassium, urea, and creatinine in an ulcer patient during 54 days. Deoxycortone, and especially liquorice, produce a gain in weight, a positive sodium and chloride balance, and a negative potassium balance, together with a decrease in hæmoglobin and a rise in venous pressure, arterial pressure, and pulse-pressure. Slight increase in blood-plasma chlorides and sodium, sharp fall in blood potassium. No influence on urea and creatinine.

patient was totally inactive. Therefore the œdema-producing factor is either glycyrrhizin or some substance precipitated with it by acid.

COMPARISON WITH DEOXYCORTONE

The effects observed were in all respects similar to those following injection of large doses of deoxycortone or of injections of adrenocorticotropic hormone (A.C.T.H.). It was now clear that succus liquiritiæ and the purified product contained a substance with an action resembling that of one of the two hormones and that this substance was active when taken by mouth.

To gain more information, we studied in one case the urinary output of sodium, potassium, and creatinine and compared the effect of a moderate and high dosage of deoxycortone with that of 20 and 45 g. of succus liquiritiæ daily.

Sodium and potassium in urine and food were determined with a flame photometer. Chloride was determined by the silver-nitrate method of Laudat (1928) and in periods of high diuresis by the method of Asper et al. (1947), checking it regularly by the silver-nitrate method. Urea-plus-ammonia was determined according to Ambard (Bots 1941), creatinine according to a modified Folin method, hæmoglobin by the alkaline-hæmatin method of Wu (1922) adapted to a photo-electric colorimeter, and total protein with the copper-sulphate specific-gravity method (Philips et al. 1943). Venous pressure was determined by the standardised Lewis method according to Allen (1948), and Borst and Molhuysen (1950). The plasma volume was determined with Congo red, the disappearance curve being determined from 10 samples of blood taken 5–15 minutes after injection.

On the 2nd day of the observation period and on the 50th day (16 days after the withdrawal of drug therapy and 6 days after the withdrawal of the sodium and chloride supplement) the patient had the same weight. We assumed that he had also on these days exactly the same amount of potassium, sodium, and chloride in his body-fluids. The average daily intake during these 49 days, based on food analyses, appeared to be (in milli-equivalents) sodium 232, chloride 165, and potassium 113, and the average output was 220, 153, and 87·5 respectively. The extrarenal loss must therefore

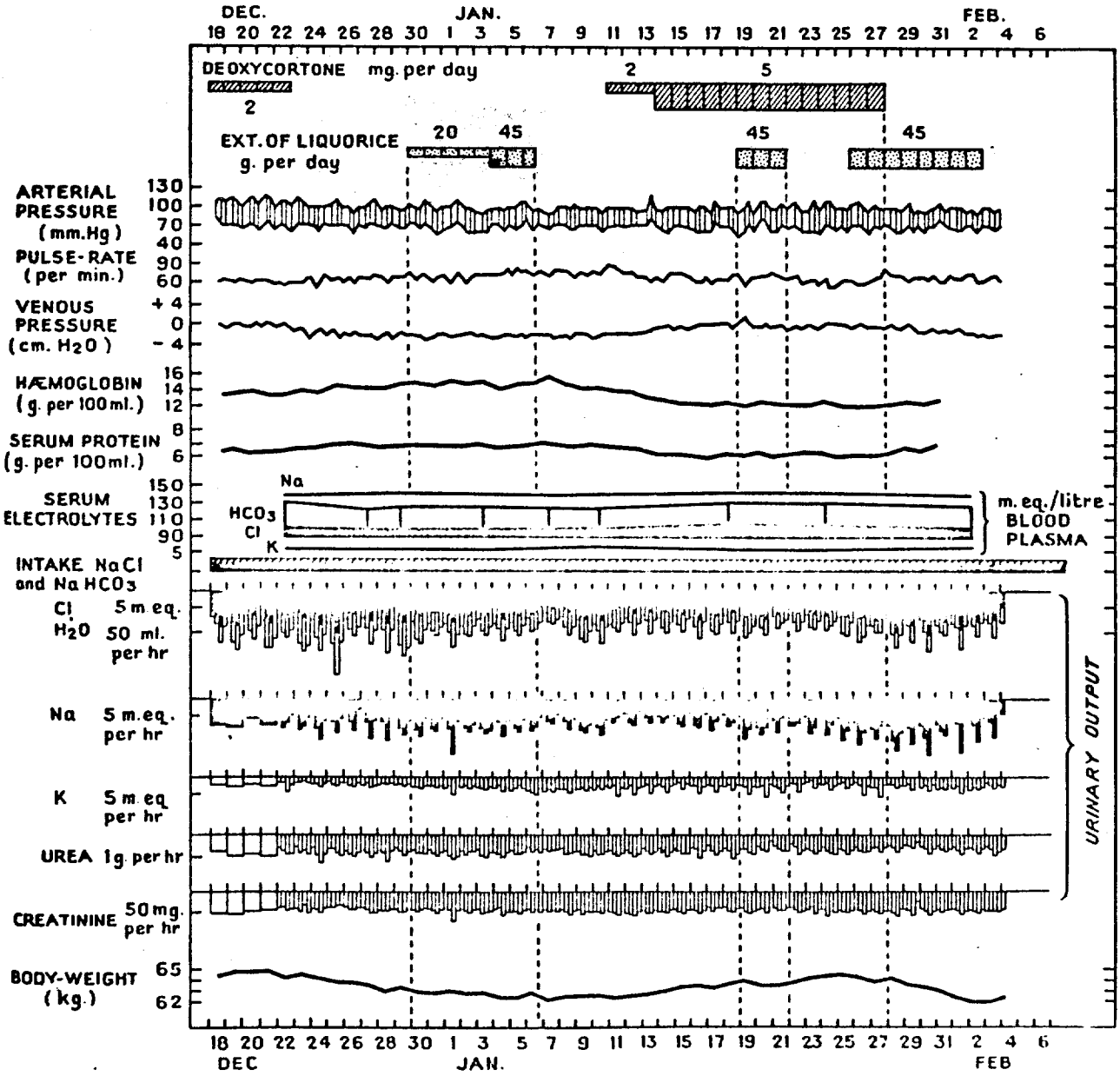

Fig. 2—Six-hourly output of water, sodium, chloride, potassium, urea, and creatinine in a patient with Addison's disease. Deoxycortone has a marked effect on weight, venous pressure, hæmoglobin, and total protein, and a slight effect on electrolyte excretion. Liquorice has no effect or a slightly opposite one.

have been sodium 12, chloride 12, and potassium 25·5 m.eq., the figures being of the magnitude that can be anticipated when the urine is carefully collected. To calculate the balance of the three electrolytes we assumed that not only the average, but also the actual, daily extra-renal losses were 12, 12, and 25·5 m.eq. respectively. The results are presented in fig. 1. The patient happened to be a "strong reactor," and the effect of 20 g. of succus liquiritiæ orally daily was quantitatively greater, though qualitatively the same, as that of two daily injections of 10 mg. deoxycortone. Both produced retention of water, sodium, and chloride, and this gave rise to the signs which overburdening of the circulation by extra-cellular fluid causes in the presence of a normal heart i.e., a fall in hæmoglobin and serum-protein, a moderate rise in venous pressure, a rise in systolic pressure, and a very marked rise in pulse-pressure, without a change in the pulse-rate, pointing to a substantial increase in cardiac output.

On Dec. 20, Jan. 25, and Feb. 1 the respective plasma-volumes were 2320, 2370, and 1980, ml., and the hæmoglobin levels 14·5, 13·4, and 16·1 g. per 100 ml. These figures show that the fall in hæmoglobin truly reflected an increase in plasma-volume.

The usual increase in output of potassium produced by deoxycortone was also seen in this case; but the

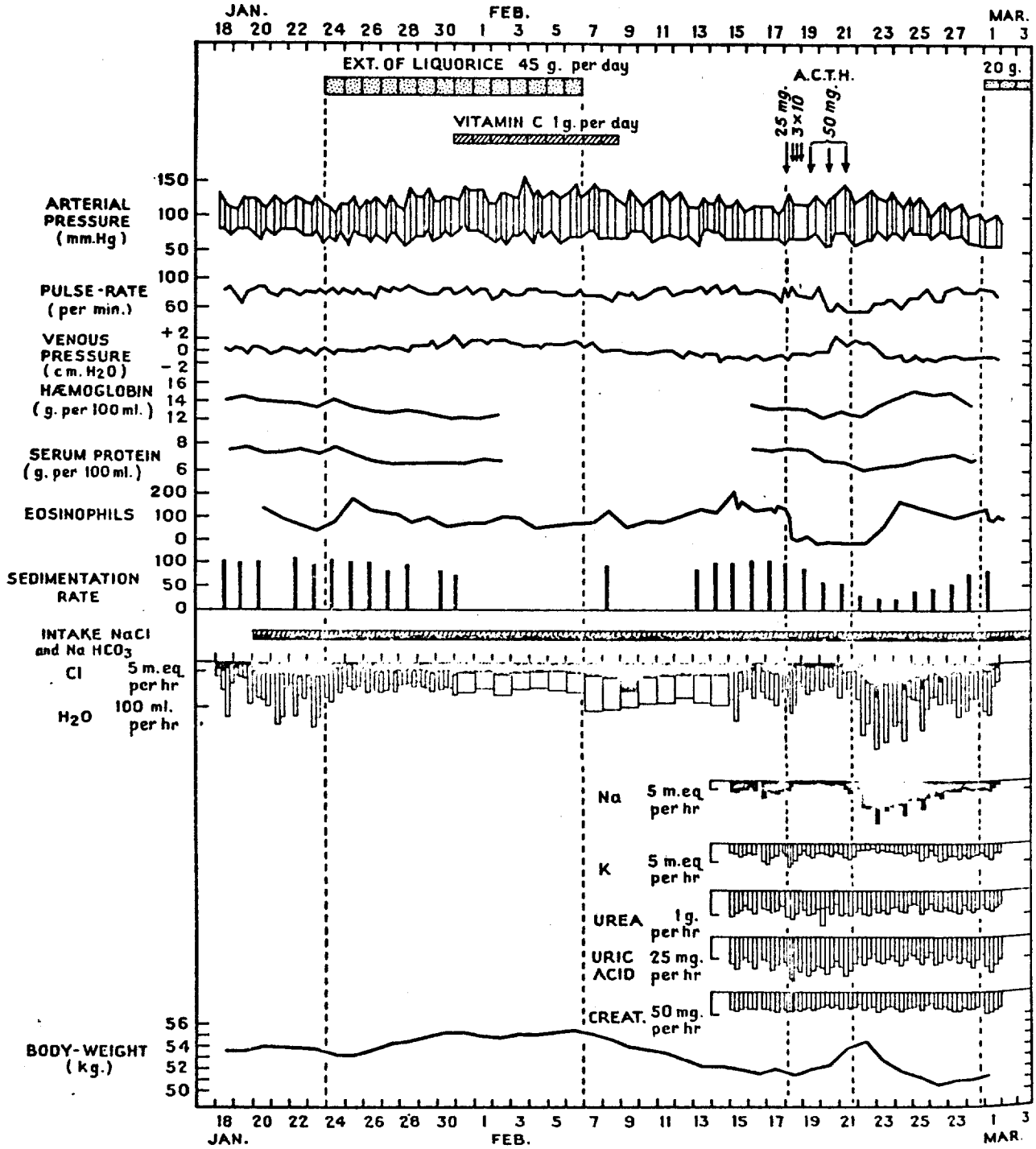

Fig. 3—Effect of liquorice on water and chloride excretion, body-weight, hæmoglobin, venous pressure, arterial pressure, eosinophil-count, and sedimentation-rate in a patient with rheumatoid arthritis. Effect of A.C.T.H. similar but more prompt and much stronger; also prompt and strong response of eosinophils and sedimentation-rate.

effect of deoxycortone was much exceeded by that of the liquorice extract, which produced a strongly negative potassium balance. At the same time the sodium balance was strongly positive, which could not be fully accounted for by the increase in extracellular fluid shown by the increase in weight and by the retention of chloride, nor by the slight increase in the plasma-sodium level. This indicates that 450 m.eq. of potassium of the cells had been replaced by sodium. Ferrebee et al. (1941), though unable to demonstrate the negative potassium balance and the positive sodium balance in dogs injected with 25 mg. deoxycortone daily, found sodium replaced by potassium in the muscles obtained by biopsy. As in Ferrebee's dogs, the potassium figures in the blood-plasma of our patients receiving liquorice were much decreased. The lowest value was 3·3 m.eq. per litre: but this patient, who was in bed, did not complain of muscle weakness. Unfortunately, electrocardiograms were not made.

During the treatment with liquorice no increase of effect could be obtained by injecting 10 mg. deoxycortone twice daily. It is highly probable that this was because the cardiac output was already greatly increased. An increase in cardiac output, unless accounted for by an increase in metabolism, is always followed by a rise in the urinary output of the three components of extracellular fluid (Borst 1938, 1941, 1948, Borst and de Vries 1950). Hence, in subjects with normal heart and kidneys, the increase of extracellular fluids during long-continued treatment with deoxycortone or succus liquiritiæ is self-limiting. The diuretic effect, postulated by several authors (Silvette and Britton 1938, Ragan et al. 1940, Ferrebee et al. 1941, Malinos et al. 1941, Gaunt et al. 1949, Roemmelt et al. 1949) as due to the continued deoxycortone injections, and even leading to "diabetes insipidus," can be explained by this mechanism.

There are a few differences in the effect of deoxycortone and succus liquiritiæ. After discontinuing deoxycortone treatment, the rebound is very pronounced and starts within 24 hours of the last injection. Discontinuance of succus therapy is not followed by so impressive a rebound: it is 10 days before the weight, hæmoglobin, and venous pressure are on the pre-treatment level; indeed, the blood-pressure remained high so long as the sodium supplement to the diet was given. The retention of potassium, starting gradually after discontinuing succus therapy, becomes almost complete in the next 5 days.

COMPARISON WITH A.C.T.H.

The above observations did not enable us to decide whether the action of the succus resembled that of deoxycortone or of A.C.T.H.; all the signs and symptoms observed in our patients can be produced by either hormone.

Accordingly we started the treatment with succus in a patient with Addison's disease. His diet was not so rigidly standardised, but we supplied daily exactly the same type of meals and fluids in the same quantities. The food was prepared without salt. The succus was of the same batch and was prepared on the same day and mixed with sodium bicarbonate and sodium chloride as in the first patient. The findings are shown in fig. 2. The patient had been on a daily injection of 2 mg. of deoxycortone for a year. After changing deoxycortone dissolved in oil to an injection of pure oil, there was no definite increase in the output of water, sodium, and chloride, nor was potassium output increased. There must nevertheless have been a slightly negative balance of extracellular fluid, for his weight went down. More pronounced and also prompt was the drop of the blood-plasma volume, as shown by the rise in the level of hæmoglobin and especially the fall in venous pressure. Then 20 g. of succus was given daily for 6 days, the dose being later increased to 45 g. a day. Response to the treatment was not apparent; if there was any, it was negative. After withdrawal of the drug, weight and plasma-volume slightly increased.

As it was possible that succus liquiritiæ only enhances the effect of the sodium-conserving hormones of the adrenals, deoxycortone and liquorice were then given simultaneously. First 2 mg. of deoxycortone, and then 5 mg., was injected daily and after equilibrium had been established, liquorice was added for 3 days. Deoxycortone produced a slight retention of chloride and sodium: the rise in weight and especially the fall in hæmoglobin and total proteins and the rise in venous pressure were striking. The liquorice was again devoid of effect: there was even evidence that it was harmful. Finally succus was given for a third period, and after 2 days we discontinued injections of deoxycortone, the patient continuing with succus only. This time the effect of the withdrawal of hormone therapy was impressive; not only venous pressure, serum-protein, and hæmoglobin reacted promptly, but also there was a significant increase in the output of chloride and especially of sodium, leaving no doubt of the total ineffectiveness of succus liquiritiæ in correcting the disturbance in the metabolism of water and electrolytes in this case of adrenal insufficiency.

Though the patient could live on a normal diet without deoxycortone injections, he did not respond to A.C.T.H. either. We injected 25 mg. of A.C.T.H. from a batch that had proved to be very active in other patients. There was no change in electrolyte or uric-acid excretion, nor was there the slightest fall in eosinophil-count (data not in figure).

Though we had observed patients with gastric ulcer who did not respond so strongly to succus as the patient whose data are presented in fig. 1, we never saw a total absence of effect or a slightly negative effect as in the patient with Addison's disease. This suggested that the action of succus liquiritiæ is mediated by the adrenals.

To determine whether the drug has a complete A.C.T.H. action a patient with chronic rheumatoid arthritis was treated with the remedy. It was a typical case of two years' duration; three courses of gold injections given in the department for rheumatic diseases had met with only temporary success. All the joints of both the patient's hands were swollen, and he could not reach a glass of water from his side-table without help. There was a stiffness of arm and shoulder, hampering all movement. The knee-joints were swollen, and fluid could easily be demonstrated. Treatment with amidopyrine 0·5 g. three times daily brought some improvement, though the sedimentation-rate did not decrease significantly. Amidopyrine was next replaced by acetylsalicylic acid 0·5 g. three times daily, and then all treatment was discontinued. Three days later we started with 45 g. of succus daily, stiffness being at its worst at that time. After 33 hours the patient thought there was some improvement and declared that he was as well as under treatment with amidopyrine. Objectively, however, the results were not impressive, though he could move a little better. His grip was not improved when measured by ergometer. Injections of ascorbic acid, given with succus treatment, were without effect.

The retention of water and sodium chloride was not as striking as in the patient with gastric ulcer described above, but there was a definite decrease in chloride and water output and the other signs of retention of extracellular fluid were also present (see fig. 3). Withdrawal of the drug was followed by a negative water and chloride balance and also by a decrease in venous pressure. At this time the patient was complaining of a severe exacerbation of his disease; and was completely incapacitated, but after a few days there was some improvement. Then we started with A.C.T.H., the first injection being 25 mg. After 6 hours he could sit unassisted, move his arms

freely, take things from the side-table, and eat without help. The situation did not change as long as A.C.T.H. was given, but 2 days after the last injection of the pituitary hormone all the incapacitating symptoms came back. After another 4 days liquorice treatment was resumed. This time there was not even any subjective improvement, though the effect on the electrolytes was as usual, and potassium in the blood fell to 3·4 m.eq. per litre (data not included in fig. 3). Uric-acid excretion, which had increased by 25% during A.C.T.H. treatment, was unchanged.

The effect of A.C.T.H. on water and electrolyte balance was much greater than the effect of succus, but the resemblance to the response of a strong reactor to succus, as demonstrated in fig. 1, is clear. The retention of sodium and chloride is more complete after A.C.T.H., but the same tendency to establish a new balance as a result of the increased blood-volume, venous pressure, and cardiac output is demonstrated by the increase of chloride and sodium excretion on the 4th day of treatment. There is, however, a difference in the prompt rebound after discontinuance of A.C.T.H. injections. The increase in the output of potassium with A.C.T.H. is lacking, but there is a retention during the "rebound." On the day we started the first course of liquorice there was a sharp, unexplained rise in the eosinophils, followed by a gradual decline that turned into a gradual rise after the treatment was stopped. A.C.T.H. brought the number down to virtually nil within 6 hours of the first injection. The rebound came 36 hours after the last injection. Resumption of liquorice was not followed by a definite decline. The sedimentation-rate (Wintrobe), which had been continuously over 100 mm. in one hour in the 4 weeks before treatment with succus, showed during the use of the drug a downward trend which was not very pronounced and could not compare with the results of A.C.T.H. treatment. The second time the same tendency towards lower figures was not observed, but there was again a rise after withdrawal of the drug.

It may be that strong reactors would have shown a greater fall in eosinophils and sedimentation-rate, and a more definite improvement. In three students, following the use of 30 g. of succus, a fall in eosinophils of nearly 20% was found, being most marked 36 hours after the succus had been taken, but not exceeding spontaneous short-lasting fluctuations seen in the same subjects. It may be that they were weak reactors for none had shown the considerable gain in weight that Revers regularly saw in specially sensitive subjects, even after the use of smaller quantities of succus.

SOME QUESTIONS

Many questions remain to be answered:

1. What is the active principle of succus liquiritiæ?
2. Are the differences in response due to differences in destruction by the juices of the digestive tract, to differences in absorption, or to considerable differences in individual sensitivity to the drug?
3. Can succus, or the active principle, be given without harm by injection, and what will be the effect?
4. Is the deoxycortone-like effect seen in eight subjects with intact adrenals but totally absent in a patient with Addison's disease, the result of stimulation of the adrenals? If it is, why is the influence of liquorice extract on eosinophils and uric-acid excretion, and on the symptoms of a patient with rheumatoid arthritis, negligible or absent?
5. Is the output of 17-ketosteroids substantially increased in patients who react to the use of liquorice by a considerable retention of extracellular fluid?

Some of these problems raise again the question of the existence and function of cortical adrenal hormones with a pure deoxycortone-like action, which, unlike 'Cortisone' and Compound F, do not affect carbohydrate metabolism, influence eosinophils and uric acid, or benefit rheumatoid arthritis.

SUMMARY

1. Observations of Revers that in a considerable proportion of normal people liquorice medication is followed by œdema are confirmed.

2. Even when no clinical œdema developed, the use of the drug resulted in retention of water, sodium, and chloride. Hæmoglobin and total serum-protein decreased; venous pressure, blood-pressure, and pulse-pressure rose considerably; and the output of potassium was much increased. The results were consistent in nine subjects with normal adrenals.

3. In a patient in whom the sodium and potassium balance was determined, a replacement of 450 m.eq. of intracellular potassium by an equal amount of sodium could be shown. The blood-potassium level fell to 3·3 m.eq. per litre.

4. The harmful effect (especially in the elderly) of potassium loss on the myocardium, which is overburdened by the increased venous pressure and by the considerable rise in blood-pressure, is emphasised. The occurrence of cardiac asthma in subjects using liquorice in sweets or in drugs is fully explained.

5. The effect of a liquorice extract by mouth is in almost all respects similar to that of injections of large doses of deoxycortone, but it lasts longer. The blood-pressure tends to remain raised after discontinuance of the drug, unless a salt-free diet is given.

6. In subjects with normal heart and kidneys the retention of water and sodium chloride during treatment with deoxycortone, A.C.T.H., or succus liquiritiæ is self-limiting, because the rise in venous pressure leads to an increase in cardiac output, which induces a saline diuresis.

7. A patient with Addison's disease not responding to A.C.T.H. did not respond positively to succus liquiritiæ: indeed there was a slight negative response.

8. A patient with rheumatoid arthritis responding favourably to A.C.T.H. showed only an insignificant improvement, or none, after the use of succus liquiritiæ.

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1. INTRODUCTION

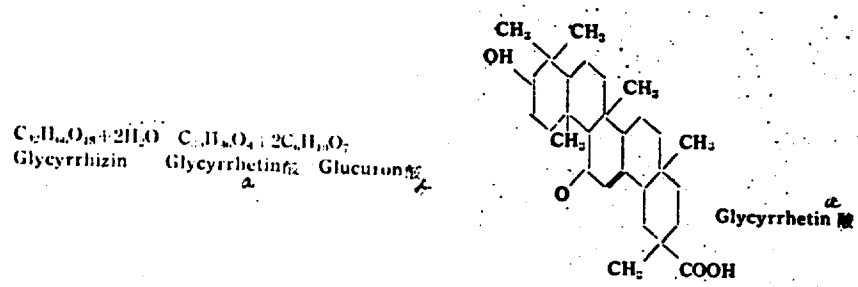
Licorice has been an important drug in the Chinese orthodox medicine for many centuries. However, its use has been confined mostly to flavoring and diluting [1,2], and its biological actions have not received much attention until recently. In 1946, Reverse [3] of Holland recognized its effect on peptic ulcers, and examined the drugs which were then used for the treatment of peptic ulcers. Discovering that their main component was licorice extract, he administered large doses of this component alone to peptic ulcer patients by the oral route. As a result, a majority of the patients indicated alleviation of subjective symptoms in short period of time, and X-ray examination confirmed dimunition of ulcers within 3 weeks. It was also noted that 20% of the patients developed edema, headaches, and shortness of breath.

Many papers on the treatment of peptic ulcers with licorice extract have since been published in Europe [5-14], calling attention to the fact that licorice extract possessed the action similar to that of DOCA, one of adrenocortical hormones. The compound has also been used for the treatment of Addison's disease [5-9].

Last year, the author treated peptic ulcer patients with licorice powder, and confirmed its therapeutic effects. He observed then that the changes in body weight, blood pressure, and the serum potassium (K) and sodium (Na) levels following the treatment exhibited a pattern

a coloration in Zimmermann reaction. From the above experimental results, glycyrrhizin evidently is different from the steroid.

Glycyrrhizin is broken into one molecule of glycyrrhetinic acid and two molecules of glucuronic acid by hydrolysis.



Keys: a, glycyrrhetinic acid; b, glucuronic acid.

The chemical structure of glycyrrhetinic acid has been investigated by L. Ruzicka [24,25], P. Kerrer [30], Karikome [27], etc., and is currently assumed to be as shown above. This is one of triterpenes, and has one OH group and ketone groups at 11 positions. It shows structural similarity to the steroid.

TABLE 1. CONSTITUENTS OF LICORICE

Glyeyrrhizin	6~14%
Liguiritin $C_{21}H_{22}O_9$(フラバノン配糖体)a	
蔗糖 b	2.4~6.5%
ブドウ糖 c	3.8%
マンニット及びアスパラギン酸 d	2~4%
その他，リンゴ酸，苦味質 e	
灰分 f	2.08~6.5%

Keys: a, flavanon glucoside; b, sucrose; d, dextrose; d, mannitol and aspartic acid; e, other constituents including malic acid, bitter component, and ash.

3. QUANTITATIVE DETERMINATION PROCEDURES

QUANTITATIVE DETERMINATION OF SODIUM

The quantitative determination of serum Na followed Albanese's

procedure [28] modified by Watanabe [29]. Since commercially sold uranium acetate is less soluble, a sample was prepared by the author.

SOLUTION 1. Uranium acetate, 10.5 g, was dissolved in 100 cc of boiling water containing 5 cc of glacial acetic acid. The solution was permitted to cool, and 25 - 40 cc of strong aqueous ammonia was added, as a result of which uranium hydroxide precipitated. Since heat was released during the reaction, the mixture was left standing to cool, then filtered with ash-free filter paper. The uranium hydroxide thus obtained was dissolved in a solution containing 50 cc of water and glacial acetic acid.

SOLUTION 2. Zinc acetate, 30 g, was dissolved in boiling water containing 1 cc of glacial acetic acid.

While boiling, the solution was combined with solution 1, and heated until it began to boil again. The solution was left standing overnight at room temperature, filtered, and combined with equal amount of 95% ethanol. The mixture was stored in ice room for 48 hours, then used. The solution was filtered prior to use.

In the quantitative determination, serum 0.5 cc, was added to 2 cc of 20% acetic trichloride, and, after shaken well, the mixture was subjected to centrifugation at 3000 rpm for 10 minutes. The supernatant, 0.5 cc, was collected, and the above reagent, added. The mixture was left standing for 2 hours. Subsequently, 3 cc of 95% ethanol was added, and the mixture was subjected to centrifugation at 2000 rpm for 5 minutes. The supernatant was discarded. After this ethanol treatment was repeated twice, 5 cc of warm water was added to dissolve the precipitates. The solution was subjected to colorimetric determination after being kept in an incubator at 37°C for one hour. The colorimetric analysis was facilitated by Leiz photoelectric colorimeter (filter, 514 mu).

QUANTITATIVE DETERMINATION OF POTASSIUM

Serum potassium was subjected to color reaction according to Yamane's method [32] in which sodium cobaltic nitrite was used, and subjected to colorimetric analysis using Hitachi photoelectric colorimeter (Hirata's modified procedure [33]).

The recently introduced flame photometers [32] (Perkin Elmer, Bechman, Lange's, etc.) have considerably simplified the measurement of Na and K. The author also employed Bechman's DU flash spectrophotometer [25-28] in part of his experiment. The standard solution contained Na at 30.0 mg/dl, K at 2.0 mg/dl, Ca at 1.0 mg/dl, and gelatin at 0.8 g/dl. The serum Na was measured with 10-fold diluted standard solution. The wavelengths used were 548 mu for Na and 768 mu for K, and the slit was 0.15 mm for both. The gases used were oxygen and acetylene, with pressures of 15 mmH_2O and 5 mmH_2O, respectively. Comparing the results with those from the chemical procedure described above, serum Na showed no significant difference, but serum K was lower by approximately 2 mg/dl. Such difference was also mentioned by other investigators [35].

TABLE 2. PENETRATION RATES OF Na STANDARD SOLUTION

標準液 Na 濃度 mg%	b 透過率 (%)					h 平均
	第1回定 c	第2回定 d	第3回定 e	第4回定 f	第5回定 g	
800	29.1	29.2	28.0	29.0	—	29.0
700	32.1	32.2	32.2	—	—	32.2
600	37.0	38.0	37.4	36.8	37.0	37.2
500	41.1	41.6	42.8	—	—	41.5
400	46.6	47.5	47.8	46.5	47.0	47.0
300	54.5	54.3	53.9	—	—	54.2
200	62.8	63.5	63.0	63.0	62.8	63.0
100	78.8	78.3	79.9	—	—	79.0

Keys: a, Na concentration of standard solution (mg/%); b, penetration rate (%); c, first measurement; d, 2nd measurement; e, 3rd measurement; f, 4th measurement; g, 5th measurement; h, mean

TABLE 3. PENETRATION RATES OF K STANDARD SOLUTION.

a 標準液	c 透過率 (%)					平均 i
b K濃度 mg/dl	第1回測定 d	第2回測定 e	第3回測定 f	第4回測定 g	第5回測定 h	
50	21.5	21.6	21.5	21.4	21.5	21.5
40	26.3	26.0	26.4	26.0	—	26.2
30	33.2	33.3	33.2	33.1	—	33.2
20	44.0	44.3	44.1	44.2	44.4	44.2
10	64.6	64.5	64.5	64.3	64.3	64.4

Keys: a, standard solution; b, K concentration (mg/dl); c, penetration rate (%); d, 1st measurement; e, 2nd measurement; f, 3rd measurement; g, 4th measurement; h, 5th measurement; i, mean

OTHER PROCEDURES

The determination of blood sugar employed Hagedorn-Jensen's method. The total serum protein level and the specific gravities of blood and serum were measured with Hitachi protein-refraktometer, and by the copper sulfate method [39], respectively.

The quantitative determination of urinary pregnandiol followed Kyoishi's procedure [40], which was originally developed by Gutermann. The serum Ca was measured by Kramer-Tisdall's method.

4. EFFECTS OF INTERNAL ADMINISTRATION OF LICORICE POWDER ON PEPTIC ULCERS

Licorice powder, 10 g/day, was internally administered to 10 gastric ulcer patients and 27 duodenum ulcer patients. Seven were hospitalized, and 37 were treated on an outpatient basis. A part of the therapeutic results are shown in Table 4. Some of these cases will be introduced below.

CASE 1. Name, Kawauchi. Male, Age, 61. Chief complaint, pains in the upper abdomen. For approximately 3 years before the present illness

was brought to medical attention, he experienced pains in the upper abdomen 2 to 3 hours after meal. Diagnosed as gastric ulcer, he was treated, and the symptom alleviated slightly. He still remained on soft diet for 2 years since normal meal caused abdominal pains. From September 1953, he experienced nausea and pains in the abdomen 2 to 3 hours after meal, and the pains began to spread to the back.

History of Present Illness. He was admitted to Kyushu Hospital University for the treatment of pyloric ulcer in September, 1943, but was discharged due to his refusal to have surgery.

He was admitted to this hospital on December 12, 1953. The findings on admission were: slightly undernourished; spontaneous pains and tenderness in upper abdomen; fecal blood reaction, positive to intermediate degree; parasitic ovum, none, X-ray test revealed a large niche in the minor curvature. The administration of licorice powder at 10 g/day, divided into three portions to be taken after meal, reduced the size of the niche (Figure 1), and led to complete dimunition in 30 days. Spontaneous pains and tenderness disappeared in 10 days, and the fecal blood test was slightly positive on the 10th day and negative on the 18th day. The gastric analysis by the caffein method revealed excessive acidity prior to the treatment, but normal acidity in 33 days (Figure 2). The patient gained weight rapidly, by 4 kg in the first week (Figure 3). The blood pressure showed a rise, from 106 / 62 mmHg prior to the treatment to 171/86 mmHg. The serum protein dropped from 7.2 g/dl to 6.7 g/dl, the serum Na increased from 335 mg/dl to 372 mg/dl, and serum K dropped from 19.3 mg/dl to 15.7 mg/dl. Hematological changes included a drop in Hb from 78% to 71% (Sahli). No appreciable change was noted in the acidocyte count of peripheral blood.

CASE 2. Name, Harada. Male. Age 45. The patient had a niche in

the minor curvature, which diminished in 16 days following internal administration of licorice powder at 10 g/day. Pyrosis eased from the 7th day of the treatment, and gastric acidity became lower. The body weight increased by 4.8 kg, the blood pressure was elevated from 104/64 to 139/84, and the total serum protein dropped from 7.3 g/dl to 6.3 g/dl (Figure 4).

The serum Na increased from 332 mg/dl to 354 mg/dl, and serum K dropped from 19.0 mg/dl to 13.2 mg/dl.

TABLE 4.

	患者c	年齢q	性r	甘草末服用期間s	X線所見u	自覚症状
a 胃潰瘍	河内d	61	♂	34日	ニッシェ消失	自発痛消失10日
	原田e	45	♂	27	●	吞酸嘈囃消失
	福本f	50	♀	23	ニッシェ縮小	腹満感消失
	久下g	54	♂	30	ニッシェ消失	自発痛消失7日
	正丸h	55	♀	14	ニッシェ不変	● 10日
	藤田i	34	♂	28	ニッシェ消失	● 1日
	久保田j	38	♂	14	ニッシェ縮小	吞酸嘈囃消失
b 十二指腸潰瘍	川上k	45	♂	25	不変	自発痛消失7日
	大田l	28	♂	14	●	●
	武藤m	26	♂	14	●	不変
	杉山n	28	♂	18	●	●
	淡路o	33	♂	20	軽快	自発痛消失7日
	小野p	18	♂	14	不変	● 1日

Keys: a, gastric ulcer; b, duodenal ulcer; c, patient; d, Kawauchi; e, Harada; f, Fukumoto; g, Hisashita; h, Masamaru; i, Fukuda; j, Kubota; k, Kawakami; l, Ota; m, Muto; n, Sugiyama; o, Awaji; p, Ono; q, age; r, sex; s, period of treatment; t, days; u, X-ray findings; v, dimunition of niche; w, reduction of niche in size; x, without change; y, without change; z, improvement; a', subjective symptom; b', spontaneous pains diminished in 10 days; c', heartburn diminished; d', abdominal fullness diminished; e', spontaneous pains diminished in 7 days; f', spontaneous pains diminished in 10 days; g', spontaneous pains diminished in 1 day; h', heartburn diminished.

similar to that following the injection of DOCA.

In subsequent experiments using animals, licorice decoction and glycyrrhizin, the main constituent of licorice, were administered to intact rabbits, nephrectomized rabbits and adrenalectomized rabbits, and changes in serum K and Na were compared with those following DOCA treatment.

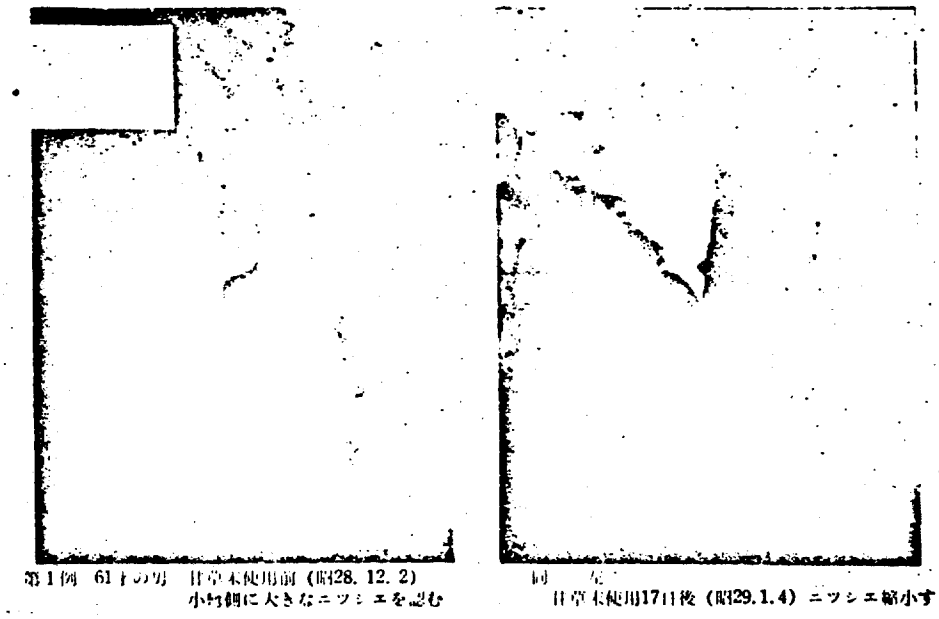
Ulcers can be produced in white rats (Shay rats) experimentally by ligating the pylorus, but licorice was found to prevent the growth of ulcer.

2. CONSTITUENTS OF LICORICE

The constituents of licorice [2,21,22,28] are listed in Table 1. Glycyrrhizin, the main constituent, is the glucoside of glycyrrhetinic acid, and is a white needle crystal with a melting point between 290 and 293°C. It is readily soluble in alcohol and alkalline solution, less soluble in ether and acetone, and sparingly soluble in water. The glycyrrhizin used in the present investigation was a product of Minophagen Institute, and had a melting point of 290°C. Its properties such as melting point were in coincidence with the above description.

The sample of glycyrrhizin used developed a reddish purple coloration in Lieberman-Buchard reaction (acetic acid anhydride was added, and a drop of concentrated sulfuric acid was gently added), and displayed a coloration in Karikome-Hashimoto's reaction at 110°C (heated with acetic trichloride crystals). Zimmerman's reaction (caustic potash was added to methadinitrobenzene alcohol) was negative. On the other hand, the steroid exhibited changes in coloration, from red to purple, then to blue, in Lieberman reaction, developed a reddish purple coloration at a temperature below 60°C in Karikome-Hashimoto's reaction, and also displayed

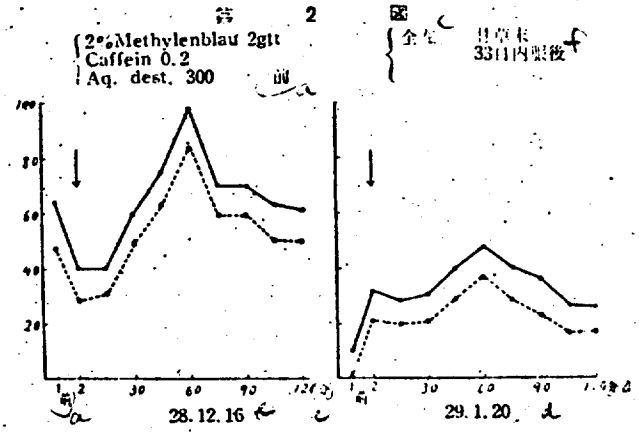
FIGURE 1.



CASE 1. Male, age 61. Prior to Treatment (December 2, 1953) A large niche observed in the minor curvature.

CASE 1. 17 days after treatment (Jan.= 4, 1954). The size of niche reduced.

FIGURE 2.



Keys: a, before treatment; b, December 16, 1953; c, minutes; d, January 20, 1954; e, same as before; f, 33 days after treatment with licorice powder.

FIGURE 3.

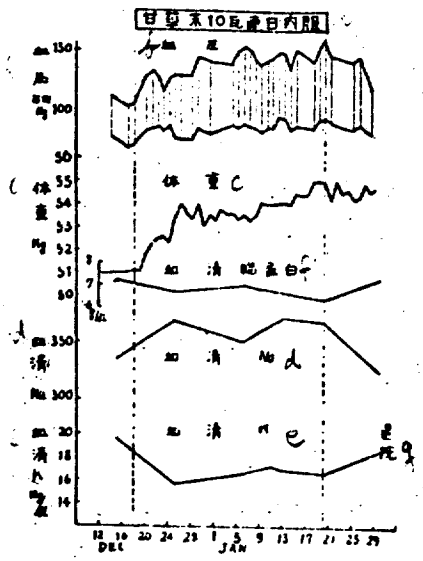
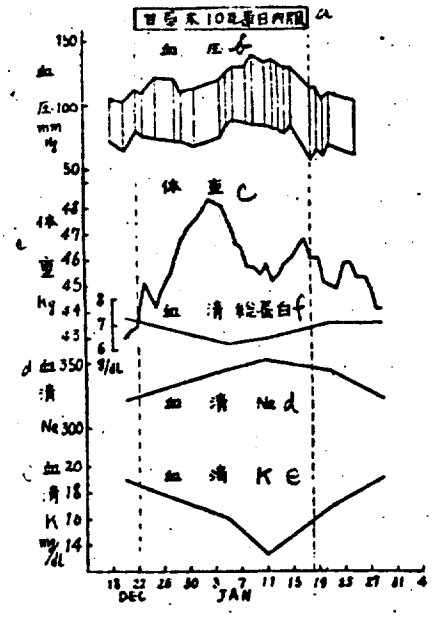


FIGURE 4.



Keys: a, internal administration of licorice powder at 10 g/day; b, blood pressure; c, body weight; d, serum Na; e, serum K; f, total serum protein; g, discharged.

Facial edema diminished upon withdrawal of licorice treatment.

CASE 3. Name, Fukumoto. Female. Age, 50.

The patient had a niche in the minor curvature. Consecutive administration of licorice powder at 10 g/day for 21 days reduced the size of the niche. The body weight increased by 2.8 kg, the blood pressure was elevated from 136/58 mmHg to 168/106 mmHg. The total serum protein level dropped from 8.1 g/dl to 7.2 g/dl. The serum Na increased from 336 mg/dl to 362 mg/dl, and the serum K decreased from 16.5 mg/dl to 12.4 mg/dl.

Averaging the three cases, the body weight increased by 3.8 kg, the systolic pressure was elevated by 46 mmHg and the diastolic pressure, by 30 mmHg. The total serum protein dropped by 0.6 g/dl, the serum Na increased by 29 mg/dl, and the serum K decreased by 4.2 mg/dl.

Including these cases, the gastric ulcer patients treated with licorice powder totalled 17, of which 13 indicated dimunition of niches, and 3, the reduction of the size of niche. One case showed no positive response to the treatment, but subjective symptoms diminished in 10 days. Of 27 duodenal ulcer cases, subjective symptoms disappeared within 7 days in 20 cases, and among the 16 cases which were subjected to X-ray examination, 2 indicated improvement.

Of 44 cases, 11, 25%, developed edema. Six of the 11 cases had gastric ulcers and 5, duodenal ulcers.

5. EXPERIMENTS WITH SHAY RATS

Many investigators produced gastric ulcers in laboratory animals by various methods: Selye used stress rats /50/; an electroshock was applied to the parasympathetic nerves /60/, Bucher induced ulcers with histamine treatment; pyloric ligation was performed /62/; ulcers were formed by allergic reaction /61/; hydrochloric acid was administered orally; ulcers were produced by nutritional disturbance; etc.

In 1945, Shay successfully produced ulcers in white rats in 17 to 19 hours by ligating their pylorus. The incidence of ulcer formation in his experiment was 94% (Shay rats). Segal reportedly attained a success rate of 100%. Many other investigators (Welfourn, Mudden, Lehman, and Risley) confirmed that ulcers could be produced by the sam method.

According to various investigations on the causative factor of experimental ulcer in Shay rat, many investigators believe that the ulcer formation is caused by the action of the gastric juice. Ishihara /71/ attributed the growth of ulcer to the circulatory disturbance in the gastric wall due to spasmodic vascular contraction.

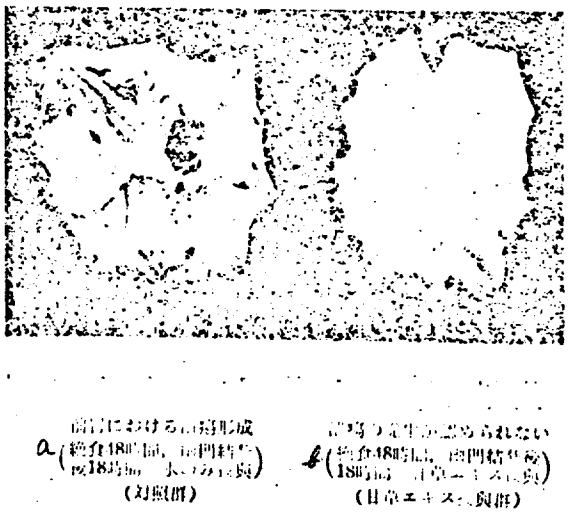
Let us review the original procedure employed by Shay. The weights

of the white rats used averaged 100 g. Since gastric contents interfers with ulcer formation, feeding was withheld for 48 hours, during which time water was given upon demand, and the rats were caged separately. Then, the rats were anesthetized with ether, and urethane was injected to the appendices. Subsequently, the pylorus was ligated while caution was taken not to tie the duodenal artery, and the abdominal wall was closed. Collodion was applied over the wound. The rats were decapitated 17 to 19 hours later, and the growth of ulcer was examined.

The author used rats weighing 100 to 150 g, and Shay's surgical procedure was followed. Shay rats, 27, were divided into three groups: the control group of 10, a group of 12 treated with licorice extract, and a group of 5 treated with glycyrrhizin. Feeding was withheld from 48 hours before surgery to 18 hours after the operation. The control group was given water upon demand from 48 hours before to 18 hours after the operation. The licorice extract group and the glycyrrhizin group were given 1% licorice extract solution and an aqueous solution of glycyrrhizin, respectively, upon demand during that period. The liquid intake during the period was approximately 30 cc, equivalent to 0.3 g of licorice extract or glycyrrhizin. Mudden reportedly achieved a success rate of 80% in growing experimental ulcers, but he also stated that the rate was increased to 93% by the injection of cortisone and ACTH. Ishihara obtained the following results by including ACTH, cortisone, and DOCA in his experiment. With ACTH, the amount of gastric juice increased slightly, and the gastric acidity was elevated. The increase in pepsin was particularly notable. With cortisone, pepsin and the acidity also increased, but not as notably as in the experiment with ACTH, and no evidence of increased ulcer production could be observed. DOCA exhibited no effect on the gastric juice or on ulcer formation.

In the author's experiment, 90% of the licorice extract group (including 4 cases to which licorice extract was injected intraperitoneally at the same time) showed suppressed ulcer production. The amount of gastric juice, the hydrochloric acid level, and the total acid level showed no appreciable difference between the control, licorice extract and glycyrrhizin groups.

FIGURE 5.



Keys: a. Ulcer formation prior to treatment (No feed was given from hrs.
48 before and 18 hours after pyloric ligation; water was given upon demand) (Control Group)

b. No ulcer was produced (No feed was given from 48 hours before and 18 hours after pyloric ligation; licorice extract was given during the period) (Licorice extract group)

6. TREATMENT OF ADDISON'S DISEASE, TETANY, PAROXYSMAL PARALYSIS, AND GERLIER'S DISEASE WITH LICORICE POWDER

Licorice powder, 10 g/day, was administered to a patient with typical symptoms of Addison's disease for 7 days, as a result of which subjective symptoms were alleviated slightly. However, no DOCA-like action on the mineral metabolism could be observed. Internal administration of licorice powder at 10 g/day to 11 cases of Addison's disease, dermatosclerosis,

urticaria, pulmonary tuberculosis, neurosis, and dwarfism resulted increased in weight, elevation of blood pressure, decreases in serum K, and increases in serum Na in 7 cases. Only 2 of them developed edema (Table 5).

TABLE 5

	a 姓	b 性	c 年齢 (才)	d 病名	e 血圧増加 (mmHg) f 最高血圧	g 最低血圧	h 体重増加 (kg)	i 甘草末10g内服期間(日)	j 浮腫出現
1	恒松	♀	21	蕁麻疹	26	0	1.6	7	(+)
2	田口	♀	25	皮膚硬症	36	0	3.3	7	(-)
3	千住	♀	26	〃	不変 k		2.0	5	(-)
4	南川	♂	35	アディソニスムス	5	0	1.7	5	(-)
5	宇都宮	♂	20	〃	10	0	1.8	7	(+)
6	城戸	♀	25	皮膚筋炎	不変 k		0.9	7	(-)
7	石橋	♀	24	神経症	〃		0.2	7	(-)
8	真壁	♀	25	肺結核	8	0	1.3	7	(-)
9	太田	♀	31	〃	10	10	2.9	7	(-)
10	渋谷	♂	25	〃	不変 k		1.0	7	(-)
11	原田	♀	17	侏儒症	12	6	1.4	7	(-)

Keys: a, name; b, sex; c, age; d, illness; e, increase in blood pressure; f, systolic pressure; g, diastolic pressure; h, body weight gain; i, period of internal administration of licorice at 10 g/day (days); j, edema; k, without change.

Under heading a: 1, Tsunematsu; 2, Taguchi; 3, Senju; 4, Minami-gawa; 5, Utsunomiya; 6, Shiroto; 7, Ishibashi; 8, Shinjo; 9, Ota; 10, Shibuya; 11, Harada.

Under heading b: 1, urticaria; 2, dermatosclerosis; 3, dermato-sclerosis; 4, Addisonism; 5, Addisonism; 6, dermatomyositis; 7, neurosis; 8, pulmonary tuberculosis; 9, pulmonary tuberculosis; 10, pulmonary tuberculosis; 11, dwarfism

The results of various tests on one of the urticaria cases are shown in Figure 6. The internal administration of licorice powder at the same dosage resulted in weight gain by 1.6 kg in 7 days, and a rise

in systolic pressure by 26 mmHg. Serum Na was increased by 52 mg/dl and serum K decreased by 2.9 mg/dl. Rashes became milder during the treatment.

Consecutive administration of licorice powder to a case of dermatosclerosis at the same dosage for 7 days produced elevation of the systolic pressure by 18 mmHg and weight gain by 3.3 kg. The serum Na increased by 34 mg/dl and the serum K decreased by 2.2 mg/dl. The peripheral blood test prior to the treatment revealed 57% of Hb, erythrocyte count of 3,200,000, leukocyte count of 7,600 and acidocyte count of 410, but 7 days of treatment changed the values to 55%, 2,600,000, 5,000, and 360, respectively. Three days after the suspension of the treatment, the leukocyte count was 7,000, and the acidocyte count, 280. In other words, the acidocyte count of the peripheral blood showed no effect of licorice treatment. Three healthy adults omitted breakfast and took 10 g of licorice powder at 8:00 a.m. The acidocyte count of the peripheral blood was measured by the direct computation method at 10:00 a.m. and the measured values at noon indicated no change.

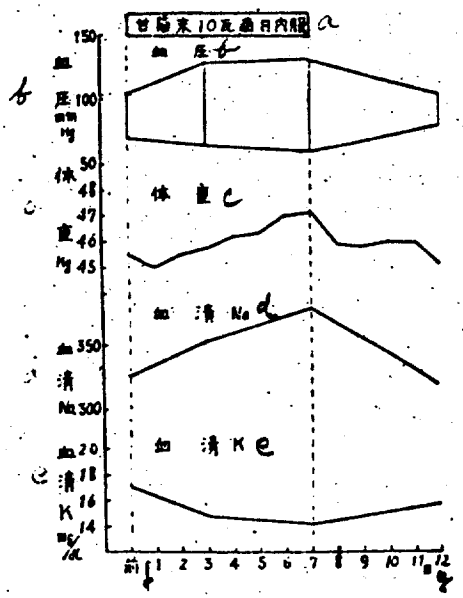
One case of Addison's disease showed serum K of 17.4 mg/dl, serum Na of 337 mg/dl, and the total serum protein level of 8.1 g/dl prior to the treatment. Seven days later, serum K decreased to 15.8 mg/dl, serum Na increased to 360 mg/dl, and the total serum protein decreased to 6.8 g/dl. The body weight increased by 1.8 kg and the systolic pressure, by 10 mmHg.

In one case of Gerlier's disease [4], licorice powder increased the frequency of paralytic seizure.

In one case of tetany, internal administration of licorice powder induced seizure. Prof. Kusunoki [42] reported that insulin injection induced tetanic seizure. Since both insulin injection and licorice powder lower the serum K level and induce alkalosis, the occurrence of seizure

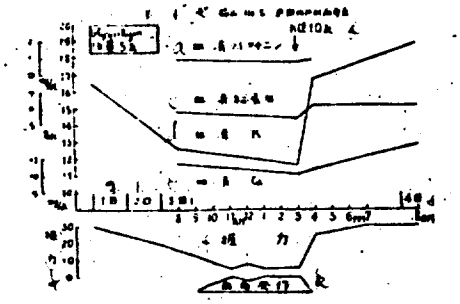
may be attributed to this particular action of licorice (Hirata).

FIGURE 6. Name, Tsunematsu. Age, 21. Female. Urticaria.



Keys: a, internal administration of licorice powder at 10 g/day; b, blood pressure; c, body weight; d, serum Na; e, serum K; f, before treatment; g, days.

FIGURE 7. Wakiyama. Age, 21. Male. Paroxysmal Paralysis.



Keys: a, serum creatinine; b, KCl, 10 g; c, total serum protein; d, serum K; e, serum Ca; f, grasping power; g, 1st day; h, 2nd day; i, 3rd day; j, 14th day; k, paralytic seizure.

In an attempt to induce seizure, crude glycyrrhizin (Yasuhara Shoten Co.: containing 91% glycyrrhizin) was internally administered to patients with paroxysmal paralysis at daily dosage of 5 g. A typical paralytic seizure occurred on the 3rd day in one case and on the 4th day in another case. During the seizure, the serum K exhibited a marked elevation and the T wave in EEC decreased [45]. When 10 g of KCl was administered internally, the paralytic seizure subsided in 1 to 2 hours in both cases. No change was noted in the acidocyte count of the peripheral blood, urinary 17-KS, or urinary uric acid-creatinine ratio at the time of seizure (Figure 7).

A patient with dermatomyositis failed to respond to cortisone injection. When licorice powder was administed at 10 g/day, neither therapeutic effect nor changes in other areas, e.g., weight, blood pressure, etc., was observed.

The treatment with licorice and cortisone in combination produced marked improvement, and a weight gain of 1.7 kg in 5 days.

7. CHANGES IN SERUM Na AND K IN NORMAL RABBITS FOLLOWING LICORICE TREATMENT

1. ORAL ADMINISTRATION OF LICORICE POWDER

Licorice powder, 2 g, was injected with a sonde to the stomach of 4 normal rabbits for 7 days consecutively (Table 7). As a result, three gained weight slightly but one remained the same. The serum K decreased in all cases, by an average of 2.9 mg/dl in 4 days, and 2.4 mg/dl in 8 days. The serum Na increased in all cases, by an average of 2.4 mg/dl in 4 days and 40 mg/dl in 8 days. Two showed decreases in the total serum protein level.

2. HYPODERMIC INJECTION OF LICORICE DECOCTION

Four mature rabbis were treated with hypodermic injection of a de decoction prepared from 5 g of licorice root (Table 8). As a result, the serum K decreased and the serum Na increased. The serum K was reduced to a notable degree in all cases, by an average of 2.4 mg/dl in 4 hours, 3.5 mg/dl in 8 hours, and 4.4 mg/dl in 24 hours. The serum Na increased in all cases, by an average of 27.5 mg/dl in 4 hours, 39.5 mg/dl in 8 hours, and 55 mg/dl in 24 hours. The decoction was injected hypodermically daily to observe changes in serum Na and K. As a result, the serum K was found to change notably on the first and second days, but the value on the third day was close to the original value. The serum Na also indicated significant increases on the first two days, but returned to the initial level on the 3rd day. These experimental findings were similar to those reported by Hirata [35] of this department who injected DOCA to rabbits daily and found that the serum K showed marked decreases but subsequently returned to the initial level despite the continuous treatment. The total protein level showed a tendency of decrease by an average of 0.7 g/dl in 24 hours.

TABLE 7. ORAL ADMINISTRATION OF LICORICE POWDER

甘草末 a	日数 c (日)	第1例 家兎 (11号) d					第2例 家兎 (12号) e				
		体重 kg e	血清K mg/dl c	血清Na mg/dl g	血液比重 h	血清比重 i	体重 kg e	血清K mg/dl f	血清Na mg/dl g	血液比重 h	血清比重 i
2 g	1	2.31	16.0	322	1.0120	1.0214	2.24	16.3	331	1.0131	1.0210
〃	2	2.42					2.18				
〃	3	2.43					2.27				
〃	4	2.44	13.2	363	1.0414	1.0204	2.28	14.5	370	1.0431	1.0212
〃	5	2.46					2.31				
〃	6	2.49					2.34				
〃	7	2.56					2.39				
中止	8	2.55	13.9	381	1.0113	1.0204	2.38	14.3	362	1.0430	1.0219
b	9	2.53					2.33				
	10	2.54									
	11	2.51	16.2	330	1.0118	1.0210					

TABLE 7 - keys: a, licorice powder; b, treatment suspended; c, No. of days; d, Case 1, rabbit (No. 11); e, body weight; f, serum K; g, serum Na; h, blood specific gravity; i, serum specific gravity; k, Case 2, rabbit (No. 12).

TABLE 8. HYPODERMIC INJECTION OF LICORICE DECOCTION

	c 第1[illegible] [illegible] (20[illegible]) [illegible] 1160 g				d 第2[illegible] [illegible] (21[illegible]) [illegible] 2200 g			
	e 血清K mg/dl	血清 Na mg/dl	g 血液比重	h 血清比重	血清K mg/dl	e 血清 Na mg/dl	g 血液比重	h 血清比重
前 a	17.5	331	1.0413	1.0221	17.2	320	1.0405	1.0219
4 時間 b	14.8	315	1.0392	1.0216	13.2	330	1.0392	1.0216
8 〃	13.3	363	1.0392	1.0215	12.6	346	1.0391	1.0215
24 〃	13.1	381	1.0384	1.0212	12.3	367	1.0389	1.0213

Keys: a, before treatment; b, hours; c, Case 1. Rabbit (No. 20); d, Case 2. Rabbit (No. 21); e, serum K; f, serum Na; g, blood specific gravity; h, serum specific gravity.

3. HYPODERMIC INJECTION OF LICORICE EXTRACT

Hypodermic injection of licorice extract (Toyo Seiyaku Co.) at 5 g to 3 normal rabbits decreased the serum K by 2.3 and 4.7 mg/dl in 2 and 4 hours, recpectively. The serum Na increased by 29.6 and 37.6 mg/dl in 2 and 4 hours, respectively. However, both serum K and serum Na returned to the initial levels 24 hours later.

8. SERUM Na AND SERUM K OF NORMAL RABBITS AFTER GLYCYRRHIZIN TREATMENT

1. INTRAVENOUS INJECTION OF GLYCYRRHIZIN

Since glycyrrhizin, the main constituent of licorice, is sparingly soluble in water, it was dissolved in an alkalline solvent.

Glycyrrhizin, 0.2 g, dissolved in physiological saline solution containing 0.15% ammonia was injected to 3 normal rabbits from the auricular vein. Physiological saline solution containing 0.15 % ammonia

was injected to control rabbits, but no marked change in serum Na or serum K was noted. Intravenous injection of glycyrrhizin produced notable decreases in serum K in all cases, by an average of 3.7 mg/dl in one hour, 4.0 mg/dl in 2 hours, and 4.4 mg/dl in 3 hours. The serum Na increased by 19.6 mg/dl in 1 hour, 33.6 mg/dl in 2 hours, and 50.3 mg/dl in 3 hours.

2. HYPODERMIC INJECTION OF GLYCYRRHIZIN

Hypodermic injection of 0.5 g of glycyrrhizin to 6 normal rabbits resulted in lower serum K and higher Na levels (Table 9). The serum K values were lower by an average of 2.9 mg/dl, 3.9 mg/dl, and 3.3 mg/dl in 4, 8, and 24 hours, respectively, than the initial level, and the serum Na, higher by an average of 35, 40, and 55 mg/dl than the initial level in 4, 8, and 24 hours, respectively. The total serum protein level was depressed in all cases, by an average of 0.8 g/dl in 24 hours.

TABLE 9. HYPODERMIC INJECTION OF GLYCYRRHIZIN

	e 2000 g			f 2100 g		
	K mg/dl	Na mg/dl	g/dl	K mg/dl	Na mg/dl	g/dl
a	16.2	339	7.0	16.8	322	6.9
b 4	12.7	372	5.95	11.1	352	6.15
c 8	12.1	369	5.8	10.7	351	5.9
d 24	12.7	391	5.9	12.8	405	5.9

Keys: a, before treatment; b, 4 hours later; c, 8 hours later; d, 24 hours later; e, Case 1. Rabbit; f, Case 2. Rabbit; g, serum K; h, serum Na; i, total protein.

However, none ofthe animals indicated changes in blood sugar level. Glycyrrhizin, 0.5 g, was injected to two normal rabbits, and blood samples were drawn 24 hours later. The measurement of serum K and Na with flame photometer revealed that in both cases, the serum K decreased,

by 3.6 mg/dl in average in 24 hours, and the serum Na increased by an average of 75 mg/dl. The serum Ca showed an average of 1.7 mg/dl decrease. DOCA, 5 mg, was injected hypodermically to two normal rabbits, and the similar results were obtained. Thus, the changes in serum K and Na as a result of hypodermic injection of glycyrrhizin followed the similar pattern as those due to DOCA injection.

3. EFFECTS OF GLYCYRRHIZIN ON URINE PREGNANDIOL

Pregnandiol (abbrviated as Preg hereafter) is a white plate crystal without physiological action, and is reported to be related to the metabolism of progesterone and DOCA. Some investigators maintain that this substance is a product of their reduction [44-46]. The currently used measurement methods include the gravimetric procedure based on Venning-Browne's method [47]. McKenna [48] and Cuyler [44] observed the enhancement of urine preg by DOCA injection. In 1944, Guterman [49, 50] formulated a method of detecting pregnancy by measuring the urinary pregnandiol, currently referred to as Guterman Test.

The author measured urine preg by Guterman's method modified by Kyoseki. Glycyrrhizin at 0.25 - 0.5 g and DOCA at 5 mg were injected to mature male rabbits by the hypodermic route (Table 10). As a result, the urine preg level was elevated. In this experiment, a modified Guterman's method was employed, but further investigation is required for the confirmation of the fact that the excreted substance was preg. The reason for the need for confirmation is that, when glycyrrhizin alone was measured by the above method, the value for Preg was clearly high.

9. INJECTION OF GLYCYRRHIZIN TO NEPHRECTOMIZED RABBITS

Nine normal rabbits were nephrectomized by bisecting the dorsal area and removing both kidneys. After the operation, the animals were

refrained from feed, and the serum K, Na and total protein levels were studied. The 9 nephrectomized rabbits were divided into a control group of 3, a group of 2 for DOCA treatment, a group of 3 for glycyrrhizin treatment, and the remaining one for licorice extract treatment. Twenty-four hours after the operation, 10 cc of 0.1% aqueous ammonia was hypodermically injected to the control group, DOCA, 5 mg, to 2 animals, and glycyrrhizin, 0.5 g (dissolved in 0.15% aqueous ammonia) to 3.

Assa result, the control group showed an average of 1.2 mg/dl increase in serum K 4 hours later. The glycyrrhizin group exhibited an average of 0.7 and 1.9 mg/dl increases in serum K, in 4 and 8 hours later, respectively. The DOCA group showed serum K values,higher than the initial value by 0.9, 2.0, and 4.0 mg/dl in average in 4, 8, and 24 hours, respectively. The rabbit treated with licorice extract showed serum K values 2.4 and 3.4 mg/dl higher than the initial level 4, and 8 hours, respectively, later. The serum Na showed gradual increases in all groups.

Thus, the decrease in serum K noted in the normal rabbits treated with hypodermic injection of glycyrrhizin or DOCA did not occur in this experiment. Hirata [35] demonstrated that insulin or adrenalin injection to nephrectomized rabbits decreased serum K but not DOCA treatment.

10. INJECTION OF GLYCYRRHIZIN TO ADRENALECTOMIZED RABBITS

Dogs and hamsters reportedly died within 10 days after bilateral adrenalectomy, and rats, within 5.7 days. Kobayashi and Kojima reported similar results. When rabbits were adrenalectomized in two separate operations in the author's experiment, most of the animals died within 1 to 10 days. The number of rabbits subjected to this experiment totalled

30. Five of them had accessory adrenal glands, and they survived over 1 months.

The author administered glycyrrhizin to adrenalectomized rabbits, and changes in serum K and Na were studied.

ADRENALECTOMY OF RABBITS The rabbit was fixed on its side, and bisected in a longitudinal direction from immediately below the costal arch at a position approximately 5 cm from the spine. Reached from the sorsal portion, the right adrenal was separated from the surrounding adipose tissue outside the peritoneum. While the organ was held with a pair of modified Shoda's forceps, the area just below the organ was tied, and the organ was carefully removed.

Seven days later, the left adrenal was removed. By removing the adrenals in two separate operations, the animal survived for 1 to 10 days. However, the serum potassium level increased abnormally, and the sodium and sugar in the blood dropped to excessively low levels.

Five cases out of 35 were found to have accessory adrenals.

A number of investigators reported that rabbits possess accessory adrenals relatively frequently.

1. EFFECTS OF UNILATERAL ADRENALECTOMY

The right adrenal was removed from 7 normal rabbits by the above procedure. When glycyrrhizin, 0.5 g, was injected hypodermically on the 7th day after the operation, all cases indicated lower serum K and higher serum Na (Table 11). The serum K showed an average of 1.4, 3.3, and 2.8 mg/dl decreases in 4, 8, and 24 hours, respectively. The serum Na increased by an average of 17, 39, and 35 mg/dl in 4, 8, and 24 hours, respectively. No change was noted in blood sugar. The total serum protein level was also depressed, by an average of 0.5 g/dl in 24 hours. The rates of change are lower than those following glycyrrhizin treatment.

TABLE 10.

	Glycyrrhizin 0.5g 皮下注射 (4例)				Glycyrrhizin 0.25g 皮下注射 (3例)			DOCA 5 mg 皮下注射 (4例)			
	第1例 (151)	第2例 (153)	第3例 (156)	第4例 (157)	第1例 (158)	第2例 (159)	第3例 (160)	第1例 (150)	第2例 (151)	第3例 (152)	第4例 (153)
a 前日尿	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
b 1日尿	(+)	(±)	(+)	(+)	(±)	(±)	(±)	(+)	(+)	(+)	(+)
c 2日尿	(±)			(±)	(±)	(−)				(±)	(±)
d 3日尿	(±)			(−)	(−)	(−)				(±)	
e 4日尿	(−)			(−)	(−)					(−)	

Keys: a, urine before treatment; b, 1st day urine; c, 2nd day urine; d, 3rd day urine; e, 4th day urine; f, glycyrrhizin 0.5 g hypodermic injection group; g, Case 1 (No. 154); Case 2 (No. 155); i, Case 3 (156); j, Case 4 (No. 157); k, glycyrrhizin 0.25 g hypodermic injection group (3 cases); 1, Case 1 (No. 158); m, Case 2 (No. 159); n, Case 3 (No. 160); o, DOCA 5 mg hypodermic group (4 cases); p, Case 1 (150); Case 2 (No. 151); r, Case 3 (No. 152); s, Case 4 (No. 153).

TABLE 11.

a 日数	c Glycyrrhizin 0.5g 皮下注射 (7例平均)				d Glycyrrhizin 0.5g 皮下注射 (7例平均)				e 0.15% 氨水 10 cc 皮下注射 (4例平均)				f DOCA 5 mg 皮下注射 (4例平均)			
	K mg/dl	Na mg/dl	蛋白 g/dl	尿素氮 mg/dl	K mg/dl	Na mg/dl	蛋白 g/dl	尿素氮 mg/dl	K mg/dl	Na mg/dl	蛋白 g/dl	尿素氮 mg/dl	K mg/dl	Na mg/dl	蛋白 g/dl	尿素氮 mg/dl
b 前	17.4	315	6.11	117	18.1	308	6.07	109	17.8	307	5.79	99	17.6	307	6.21	107
1	16.0	332	6.05	118	16.7	321	5.82	101	18.2	301	5.69	101	17.1	316	5.73	91
8	11.1	351	5.80	121	15.7	310	5.32	100	18.5	300	5.70	100	15.2	317	5.69	93
21	14.6	319	5.70	113	16.9	333	5.67	109	19.6	293	5.69	100	15.1	335	5.66	102

Keys: a, time; b, before treatment; c, Group treated with hypodermic injection of 0.5 g glycyrrhizin 7 days after unilateral adrenalectomy (average of 7); d, group treated with hypodermic injection of 0.5 g glycyrrhizin 2 days after bilateral adrenalectomy (average of 7); e, group treated with hypodermic injection of 0.15% ammonia water, 10 cc, 2 days after bilateral adrenalectomy (average of 4); f, group treated with hypodermic injection of 5 mg DOCA 2 days after bilateral

TABLE 11. Keys-continued.

adrenalectomy (average of 4); g, serum K; h, serum Na; i, total protein; j, blood sugar.

2. EFFECTS OF BILATERAL ADRENALECTOMY

When the rabbits were treated with hypodermic injection of 0.15 % aqueous ammonia, 10 cc, only on the 2nd day after bilateral adrenalectomy, the serum K increased and the serum Na decreased (Table 11). By average of 4, the serum K was higher by 0.4, 0.7, and 1.6 mg/dl 4, 8, and 24 hours later, respectively, and the serum Na was lower by 3, 7, and 14 mg/dl 4, 8, and 24 hours later, respectively. When glycyrrhizin, 0.5g, was injected hypodermically 2 days after the operation, the serum K dropped by 1.4, 2.4, and 1.3 mg/dl in 4, 8, and 24 hours, respectively, from the initial level, the values being an average of 7, and the serum Na increased by 13, 32, and 24 mg/dl in 4, 8, and 24 hours, respectively. No change was noted in blood sugard, and the total serum protein level was depressed by 0.4 g/dl.

Hypodermic injection of DOCA at 5 mg on the 2nd day after bilateral adrenalectomy decreased the serum K and increased serum Na. By average of 4, the serum K showed decreases by an average of 0.5, 2.4, and 2.5 mg/dl in 4, 8, and 24 hours, respectively, and the serum Na increased by 9, 10, and 28 mg/dl in 4, 8, and 24 hours, respectively. In other words, both glycyrrhizin and DOCA decreased serum K and increased serum Na in adrenalectomized rabbits. However, the rates of decrease or increase in this experiment were lower than those after unilateral adrenalectomy of normal rabbits.

The effects of daily injection of glycyrrhizin and DOCA were also investigated (Table 12). First of all, blood was drawn from bilaterally

adrenalectomized rabbits daily without treatment, and changes in serum K, Na and total protein, and blood sugar were examined. The serum K exhibited gradual increases, whereas serum Na showed a tendency of decrease. The blood sugar level became lower, but the protein level increased slightly.

TABLE 12. CHANGES IN THREE BILATERALLY ADRENALECTOMIZED RABBITS

	(85号) 第1例 2020 g				(100号) 第2例 2040 g				(79号) 第3例 1990 g			
	血清K mg/dl	血清Na mg/dl	総蛋白 g/dl	血糖 mg/dl	血清K mg/dl	血清Na mg/dl	総蛋白 g/dl	血糖 mg/dl	血清K mg/dl	血清Na mg/dl	総蛋白 g/dl	血糖 mg/dl
術前	16.0	316	6.63	134	16.2	331	6.46	124	15.8	314	6.23	133
1	17.5	339	4.82	119	17.5	326	6.06	104	17.9	307	6.22	114
2	35.0	306	5.12	107	23.6	293	6.55	104	19.6	305	6.42	100
3	40.8	298	6.19	108	26.9	274	6.60	81	19.9	301	6.69	119
4				死亡				死亡	20.3	299	6.66	112
5									21.3	286	6.95	119
6									21.8	273	6.90	76
7												死亡

Keys: a, before treatment; b, day(days); c, (No. 85) Case 1; d, serum K; e, serum Na; f, total protein; g, blood sugar; h, Case 2 (No. 100); i, Case 3 (No. 79); j, died.

Then, 4 bilaterally adrenalectomized rabbits were treated with hypodermic injection of 0.5 g glycyrrhizin on the 2nd and 3rd days after the operation. As a result, all four animals indicated decreases in serum K and increases in serum Na. The serum K and Na values on the 4th day after operation were an average of 3.6 mg/dl lower and an average of 22 mg/dl higher, respectively, than the initial levels.

When two bilaterally adrenalectomized rabbits were treated with DOCA-5 mg hypodermic injection on the 2nd and 3rd days after the operation, the serum K and Na values on the 4 th day after operation showed a decrease and an increase, respectively.

11. DISCUSSION

Revers was the first investigator who reported that licorice extract was effective in the treatment of peptic ulcers. He also noted edema following such treatment in 20% of his patients. Edema first occurred in the fact, apreading to the limbs [4]. Based on his report, the investigators of Utrecht University [75] conducted animal e periments and obtained the following results.

Licorice extract possessed strong antispasmodic power, and suppressed the growth of experimental ulcer in white rats and guinea pigs, but the main constituent of licorice, glycyrrhizin, possessed no action of this nature. The author administered licorice extract or glyzyrrhizin to Shay rats, and found that glycyrrhizin failed to prevent the growth of ulcer in laboratory animals. However, ulcers could be prevented by licorice extract.

Subsequently, Molhuysen, Nelemans, Schurrmans, Harz, Irle, Wilder, etc. stated that licorice extract was effective in arresting gastric ulcers. Schulze and Franke brought complete recovery to 24 cases of gastric ulcer and 4 out of 12 cases of duodenal ulcer, and alleviation to 8 cases of duodenal ulcer by using this compound for 3 to 4 weeks. They also observed its DOCA-like action. More recently, Schulze reported that licorice extract was effective in healing relatively fresh gastric and duodenal ulcers but not chronic cases of duodenal ulcer and gastritis, and that the side effects of edema and hypertension could be controlled by reducing daily salt intake to 5 g. He also administered the ammonium salt of crude glycyrrhizin, and observed positive responses. Thus, he assumed that the constituent of licorice which was effective to gastric ulcer was glycyrrhizin. Holler conducted an experiment with excised white rat intestinal tubes, and demonstrated that licorice extract

possessed papaverine-like action but such action had the strength only 1/1000 that of papaverine. Observing that the licorice extract from which glycyrrhizin had been removed improved ulcers, he assumed that the effective constituents did not include glycyrrhizin. In his investigation on the movement of white rat intestines following Magnus' method, Higo found that licorice decoction inhibited the movement but not glycyrrhizin.

Gray and Gerry assumed that the stimuli which induced ulcers were transmitted to the stomach by two routes, i.e., through the vagus nerve and by way of the adrenal cortex, of which he particularly emphasized the latter. He observed increased gastric acidity and pepsin following the administration of ACTH or cortison, and subsequent aggravation of gastric ulcers. On the other hand, DOCA, one of adrenocortical hormones, is reported as effective toward gastric ulcer by many investigators. The therapeutic effect of DOCA on gastric ulcer has been substantiated by Broicher /85/, Kohler, Hoffmeister, Hormuth, etc., whereas such value of DOCA was denied by Shilling, Boch, etc. However, the author observed that licorice possessed DOCA-like action and was effective to gastric ulcer.

Graber and Bombskov stated that follikelhormon and V.E., respectively, stimulated the blood circulation in the gastric mucuous membrane and improved the gastric ulcer. Similarly, Costello reported that licorice extract possessed estrogen-like action.

Klimpel held that licorice extract lowered gastric acidity. In the treatment of peptic ulcers with licorice powder, the author also observed decreases in gastric acidity. At the same time, one of ulcer patients was subjected to gastric analysis with a test solution prepared from caffein and licorice extract. Under this condition, the acidity remained the same as in the test using caffein only.

In 1953, Schulze obtained the following results from his animal experiment. Following consecutive administration of licorice extract, the size of adrenal cortex was reduced, and thyloid activity, slightly stimulated, which in turn activated the production of thyreotropic hormones by the anterior pituitary to a marked degree. These actions are similar to those exhibited by DOCA. Furthermore, the administration of table salt prolonged the life of adrenalectomized animals. Similarly, the life of animal could be prolonged by glycyrrhetinic acid. However, Card /95/ failed to observe life-prolongation effect of licorice extract or glycyrrhetinic acid in adrenalectomized rats.

In 1950, Molhuysen administered licorice extract to 10 patients, and 9 of them indicated abnormal retention of moisture and Na, hypertension, and depletion of K, the symptoms normally following the administration of DOCA. However, licorice extract obviously induced no effect on a case of Addison's disease. These experimental findings point to a possibility that licorice possesses the ACTH-like action that stimulates the adrenal cortex. However, ACTH is known to induce reactions specific to individual species. Molhuysen and Schulze denied that the action of licorice exhibited any resemblance to that of ACTH, by the grounds that licorice extract failed to reduce the acidocyte count in peripheral blood, it elevated the urinary uric acid vs. creatinine ratio, and its was not effective to rheumatoid arthritis. Borst observed that licorice decoction exerted DOCA-like action in healthy adults, and Grön, Card, Parrisch, etc. found licorice decoction to be effective in the treatment of Addison's disease. Grön and other investigators obtained positive results from the treatment of Addisonism with glycyrrhizin, and Card and Pelser, from the treatment of the same with glycyrrhetinic acid, a product of the hydrolysis of glycyrrhizin. Thus, they believed that the DOCA-like action

was derived from glycyrrhetinic acid, and maintained that, in the treatment of Addison's disease, the abnormal salt metabolism could be treated with licorice extract and glycyrrhizin instead of DOCA. Pelser and Rowland obtained favorable results from the prolonged administration of licorice extract in the treatment of Addison's disease. Such treatment caused neither cardiac or renal disturbance nor abnormality in androgen metabolism. Borst used licorice extract to three cases of Addison's disease, but observed no DOCA-like action. When the extract was combined with 10 mg cortisone, the patients began to show improvement. He also stated that the effect of cortisone appeared at small doses if licorice extract was also administered, and the two were synergic. Ogata obtained favorable therapeutic effects after administering licorice powder at 15 g to two cases. Schulze used licorice extract on two cases of Addison's disease, and observed its DOCA-like action.

In regard to other areas of application, Schulze treated rheumatoid arthritis and bronchial asthma with licorice powder, but failed to observe any positive effect. Hart administered glycyrrhizin to 11 cases of rheumatoid arthritis and Schwarty, to the patients who had undergone bilateral adrenalectomy, and they also observed some degree of synergic action between glycyrrhizin and cortisone.

As stated above, the clinical and experimental results from the treatment of Addison's disease with licorice extract are contradictory. This may be due to the difference in the type of licorice and dosage in different experiments.

In the present investigation, the author observed DOCA-like action of licorice powder in all patients except one case of Addison's disease. It was noted that the weight gain and changes in other areas following the treatment varied greatly according to the individual even in those

with normal adrenal activity. Borst has ascribed such variation to the difference in individual homeostatic mechanism. Glycyrrhizin is also known to possess detoxication action, as has been widely recognized in Japan.

12. CONCLUSIONS

The results of the experiment are summarized as follows.

1. The internal administration of licorice powder to 44 cases of peptic ulcer patients produced favorable results.

2. The administration of licorice extract to Shay rats checked the growth of ulcer in the laboratory animals. Crude glycyrrhizin was found without effect.

3. The treatment of 11 cases of internal disorders with licorice powder for 5 to 7 days resulted in increases in body weight, the elevation of blood pressure, decreases in serum K, and increases in serum Na in 7 cases.

4. Such DOCA-like action failed to occur in one case of Addison's disease.

5. Licorice powder and glycyrrhizin induced spasms and paralytic seizures in one case of tetany, 2 cases of paroxysmal paralysis, and 1 case of Gerlier's disease.

6. The injection of glycyrrhizin or licorice decoction to normal rabbits and adrenalectomized rabbits resulted in lower serum K and higher serum Na. DOCA injection resulted the same.

7. Hypodermic injection of glycyrrhizin to nephrectomized rabbits exerted no effect. DOCA also failed to exhibit its action in nephrectomized rabbits.

8. The injection of glycyrrhizin to adrenalectomized rabbit decreased

the serum K level and elevated the serum Na level, but the rate of change was less than that in normal rabbits.

9. The hypodermic injection of glycyrrhizin to normal rabbits increased urinary pregnandiol.

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Short Communications

SC 23009

Effects of glycyrrhizin and glucuronic acid on hemoglobin and on the activity of certain enzymes

Glycyrrhizin obtained from *Glycyrrhiza glabra*[1] is a glycoside consisting of glycyrrhetinic acid and glucuronic acid in the proportion 1:2. The glycoside is of physiological interest since it shows detoxification of alcohol and inhibition of the promoting action of oxytocin. The study reported here was undertaken in order to observe its chemical effects on hemoglobin.

Hemoglobin was obtained from Nutritional Biochemical Corporation, U.S.A. Urea-denaturated hemoglobin was prepared by the method of ANSON[2]; all experiments were carried out on the denatured protein. Glycyrrhizin and glucuronic acid (Chūgai Pharmaceutical Company Ltd.) were kind gifts from Dr. O. MINOUCHI of Minofagen Pharmaceutical Company Ltd. Crystalline proteinase[3] was obtained from Nagase Sangyo Company Ltd.; it was used as a 1 mg/ml aqueous solution. All other materials used were of reagent grade. Fresh dog spleen was obtained and 1 g wet weight of

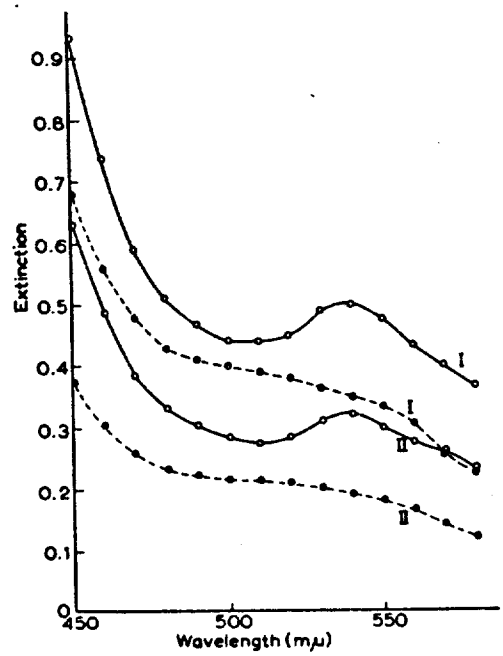

Fig. 1. Absorption spectra of hemoglobin in the presence of glycyrrhizin and glucuronic acid in the visible region. O—O, Glycyrrhizin solutions; hemoglobin concentration 0.10% (Curve I) and 0.10% (Curve II), glycyrrhizin concentration $5.7 \cdot 10^{-3}$ M. Hemoglobin alone gave the same spectrum. ●- - -●, Glucuronic acid solutions; hemoglobin and glucuronic acid concentrations the same as above.

spleen was homogenated with 10 ml cold water; the homogenate was centrifuged and the supernatant was used. Enzymic activity was measured by the method of FOLIN AND CIOCALTEU[4]. A Beckman DU spectrophotometer was used with 1-cm quartz cells.

A qualitative study was made of the heat-precipitation of urea-denaturated hemoglobin. A solution containing only the protein showed no precipitation on heating; but on the addition of glycyrrhizin (final concentration $1.2 \cdot 10^{-3}$ M) the protein precipitated completely. The addition of glucuronic acid (same final concentration) did not cause the protein to precipitate. The protein precipitated on addition of mercuric chloride, but not if the mercuric chloride was added together with glycyrrhizin or glucuronic acid in the same concentration as before. On the other hand, the precipitation effects of ammonium sulfate, trichloracetic acid, concentrated inorganic acids, acetone, etc., were not affected by the simultaneous addition of glycyrrhizin or glucuronic acid.

TABLE I

EFFECT OF CONCENTRATION OF GLYCYRRHIZIN AND GLUCURONIC ACID ON ENZYME ACTIVITY

Glycyrrhizin and Glucuronic acid concentration (mmoles/l)	$E^{1\,cm}_{650\,m\mu}$			
	Glycyrrhizin		*Glucuronic acid*	
	Proteinase	*Spleen homogenate*	*Proteinase*	*Spleen homogenate*
4	—	—	0.68	0.53
2	0.32	0.15	0.59	0.42
1	0.37	0.17	0.55	0.41
0.5	0.38	0.23	0.50	0.40
0.25	0.39	0.27	0.47	0.39
0.13	0.40	0.32	—	—
H_2O	0.45	0.38	0.45	0.38

Changes in the absorption spectra of denatured hemoglobin on addition of glycyrrhizin or glucuronic acid were studied in the visible region, as shown in Fig. 1. From the figure it can be seen that the absorption band of hemoglobin (0.16 %) at 540 mμ disappeared in the presence of $5.7 \cdot 10^{-2}$ M glucuronic acid, but that the same concentration of glycyrrhizin had no effect.

The effects of glycyrrhizin and glucuronic acid on the enzymic activities of proteinase and the spleen homogenate system were studied with denatured hemoglobin as substrate. The results are given in Table I. From the table it can be seen that both activities were inhibited by glycyrrhizin and that both were increased by glucuronic acid. It is of interest that the effects of glycyrrhizin and glucuronic acid are contrary to each other in spite of the fact that glycyrrhizin contains two glucuronic acid residues.

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THE FATE OF TRITIUM-LABELLED β-GLYCYRRHETIC ACID IN THE RAT

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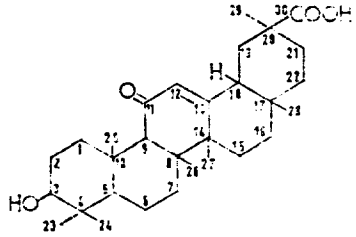
Tritium-labelled β-glycyrrhetic acid was prepared and administered orally, subdermally and intraperitoneally to male and female rats. Most of the radioactivity was excreted in the faeces, only traces being found in the urine. Administered to rats with biliary cannulae most of the radioactivity was excreted in the bile. Three metabolites of β-glycyrrhetic acid have been detected in the bile, but these have not yet been identified.

β-Glycyrrhetic acid (3-hydroxy-11-oxo-18-β-olean-12-en-30-oic acid) is a triterpenoid which occurs as the diglucuronide, glycyrrhizic acid, in liquorice root. β-Glycyrrhetic acid and its derivatives have been used in the treatment of gastric ulcer (Doll, Hill, Hutton and Underwood, 1962) and of dermatitis (Colin-Jones, 1960) and it has been shown to reduce hypercholesteraemia (Shibata, 1961). The metabolism of orally administered tritium-labelled glycyrrhetic acid and monoammonium glycyrrhizate in man has been studied by Carlat, Margraf, Weathers and Weichselbaum (1959), who suggested that both these compounds were poorly absorbed from the gastrointestinal tract, since most of the material was excreted in the faeces. We have now studied the fate of tritium-labelled β-glycyrrhetic acid in rats and have found that the compound is absorbed well from the alimentary canal. Most of the radioactive material is excreted in the bile and is then eliminated in the faeces, mainly as metabolites.



β-Glycyrrhetic Acid

Experimental

Materials

β-Glycyrrhetic acid randomly labelled with tritium was prepared from tritiated water and unlabelled β-glycyrrhetic acid, m.p. 284 − 7°, $[\alpha]_D^{19}$ + 163 ± 1° (*c*, 1 in $CHCl_3$) by the method of catalytic exchange (see Gould, 1958). 3-Acetyl-β-glycyrrhetic acid (1·2 g.) together with 660 mg. of tritiated water (2·5 c, Radiochemical Centre, Amersham), 3·3 ml.

acid and 67 mg. of pre-reduced platinum oxide catalyst (Adams, Voorhees and Shriner, 1941) were heated at 127° for 72 hr. in a sealed tube. The product, dissolved in 5 ml. water and 135 ml. of a 2·5 per cent w/v solution of potassium hydroxide in methanol, was then hydrolysed by refluxing under nitrogen for 2 hr. The hydrolysed solution was acidified with 100 ml. 2N hydrochloric acid and the white precipitate of tritium-labelled β-glycyrrhetic acid which formed was extracted into 150 ml. chloroform. The aqueous layer was further extracted with two 50 ml. portions of chloroform and the combined extracts washed twice with 100 ml. water, dried over anhydrous sodium sulphate and the solvent evaporated. The crude product (yield, 1·07 g., m.p. 270–275°) was recrystallised from a mixture of methanol and chloroform to give pure tritium-labelled β-glycyrrhetic acid (0·8 g.), m.p. 281–5 $[\alpha]_D^{20}$ + 169° ± 2° (c. 1 in $CHCl_3$), 3 mc g. The product gave a single spot when run on a paper chromatogram by descending chromatography on Whatman No. 1 paper in a solvent consisting of light petroleum (b.p. 80–100°): methanol: water (5:4:1 by vol.) (Bush, 1952). The infra-red spectra was identical with that of authentic β-glycyrrhetic acid.

METHODS

Animal experiments. ^{3}H-β-Glycyrrhetic acid was administered orally to albino rats (weighing *ca.* 150 g.) as an aqueous suspension or as a solution in arachis oil, and intraperitoneally as a solution in propylene glycol.

Determination of radioactivity. In preliminary experiments, the faeces, urines and tissues were dissolved in formamide (Kinnory, Kanabrocki, Greco, Veatch, Kaplan and Oester, 1958) and the radioactivity counted in a Panax refrigerated scintillation counter type SC-LP at 7°. A solution of a mixture of naphthalene, 2,5-diphenyloxazole (PPO), 1,4-bis-[2′-(5′-phenyloxazole)]-benzene (POPOP) (800:50:1, by weight) in toluene: ethanol: dioxane (5:4:3, by volume) (TED/5 Panax) was used as phosphor. In subsequent experiments, tissues, faeces, urine and bile were dried in cellophane packets under infra-red lamps, and then ignited in an atmosphere of oxygen (Kelly, Peets, Gordon and Buyske, 1961). The ^3H_2O formed by the combustion was dissolved in TED 5 phosphor (Panax) and the solution counted as before. Results obtained by these two different methods showed no significant differences, but the latter method eliminated the high tissue blanks observed in the formamide method.

Biliary cannulation. To investigate the possibility of biliary excretion of glycyrrhetic acid or its metabolites, ^{3}H-β-glycyrrhetic acid was administered orally or intraperitoneally to albino rats after surgical insertion of a polythene cannula into the common bile duct (Stewart and Harrison, 1961). The rats were kept in wire restraining cages (Bollman, 1948) and were allowed free access to an aqueous solution of glucose (5 per cent w/v)-saline (1 per cent w/v), with or without addition of bile salt (0·04 per cent w/v). The bile, urine and faeces were collected at intervals over a period of three days and together with tissues, were combusted to ^3H_2O and the radioactivity measured.

Chromatography of metabolites. The bile obtained from cannulated rats dosed with ^{3}H-β-glycyrrhetic acid was first chromatographed on Whatman No. 1 paper in solvent A, i.e. chloroform : acetic acid : water (2 : 1 : 1 by vol.). Radioactive material remaining at the origin was eluted and further chromatographed on Whatman 3 MM paper in solvent B, i.e. the lower phase of 70 per cent v/v aqueous acetic acid : 1,2-dichloroethane : n-butanol (10 : 9 : 1 by vol.) by the descending technique (Sjovall, 1955). The chromatograms were cut into strips and the positions of the radioactive metabolites determined by absorption in ultra-violet light and by combusting 1 cm. bands of the paper and counting the ^3H_2O produced.

Estimation of unchanged β-glycyrrhetic acid in faeces. The unchanged β-glycyrrhetic acid in the rat faeces was determined by a reverse isotope dilution procedure. β-Glycyrrhetic acid (250 mg.) was added to dried faeces (1·0 g.) and the mixture extracted with chloroform in a Soxhlet apparatus for 1·5 hr. The chloroform extract was concentrated to 5 ml. and an aliquot (one-fifth) was chromatographed on Whatman 3 MM paper in solvent A. The area of the chromatogram between R_F 0·6–1·0 was cut out and eluted with chloroform : methanol (9 : 1 by vol.) and the eluate re-chromatographed on Whatman No. 1 paper in light petroleum (b.p. 80–100°) : methanol : water (5 : 4 : 1) (solvent C). The area between R_F 0·0–0·17 of the second chromatogram was cut out and eluted as before and the eluate evaporated to dryness (52 mg.). An aliquot (ca. 1 mg.) of the residue was ignited and the radioactivity of the water of combustion determined.

TABLE I

PAPER CHROMATOGRAPHY OF THE GLYCYRRHETIC ACIDS AND SOME DERIVATIVES

Chromatograms were run on Whatman No. 1 paper in solvent A : chloroform : acetic acid : water (2 : 1 : 1 by volume); B : the lower phase of 70 per cent v/v aqueous acetic acid : 1,2-dichloroethane : n-butanol (10 : 9 : 1) (Sjovall, 1956); and C : light petroleum (b.p. 80–100°) : methanol : water (5 : 4 : 1, by volume) by descending technique (Bush, 1952).

Compound	R_F value in solvent		
	A	B	C
β-Glycyrrhetic acid	0·98	0·95	0·1
α-Glycyrrhetic acid	1·0	1·0	0·0
3-Keto-β-glycyrrhetic acid	1·0	1·0	0·0
3-Acetyl-β-glycyrrhetic acid	1·0	1·0	0·57
Ammonium glycyrrhizinate	0·06	0·60	0·0
Metabolite I	0·0	0·40	0·0
Metabolite II	0·0	0·46	0·0
Metabolite III	0·44	0·77	0·0

Chromatography in solvent A separates β-glycyrrhetic acid from its metabolites, which are found at the origin and at R_F 0·1–0·4 (see Table I) and chromatography in solvent C separates β-glycyrrhetic acid from less polar compounds naturally present in faeces.

RESULTS

After administration of an oral dose of 60 mg./kg. of finely ground ^{3}H-β-glycyrrhetic acid suspended in water (3 ml.) to female rats, an

average of 86 per cent of the administered radioactivity was recovered in [illegible] days, 83 per cent being found in the faeces, 4 per cent in the liver and [illegible] per cent in the urine (see Table II). After sub-dermal injection of an aqueous suspension of ^{3}H-β-glycyrrhetic acid (60 mg./kg.) to female rats, an average of 74 per cent of the administered radioactivity was recovered in 5 days, 73 per cent being in the faeces and 1 per cent in the urine. Of the radioactivity present in the faeces, only 7·4 per cent was found to be β-glycyrrhetic acid when the triterpenoid was administered orally, and only 5·2 per cent when it was administered sub-dermally (see Table II). The levels of radioactivity found in the liver and gastrointestinal contents after oral administration were found to vary with the duration of the experiment in a manner suggestive of an entero-hepatic circulation of β-glycyrrhetic acid.

TABLE II

FATE OF ^{3}H-β-GLYCYRRHETIC ACID IN RATS

Expt. No.	Sex	Duration of expt. (days)	Dose per cent of radioactivity found in: Faeces	Urine	Liver	Total per cent of dose accounted for
Oral doses of 60 mg./kg. in aqueous suspension:						
1	F	1	70*	—	7·5	78
2	F	1·5	112*	—	2·6	115
3	F	2	82**	1·0	—	83
4	F	3	56	1·2	—	57
5	F	3	94	—	3	100†
6	M	2	46*	—	9·3	56
Sub-dermal doses of 60 mg./kg. in aqueous suspension:						
7	F	5	84‡	0·8	—	85
8	F	5	62	1·2	—	63

* Also includes gastrointestinal contents.
† Includes 3 per cent found in gastrointestinal contents.
** 7·4 per cent present as β-glycyrrhetic acid.
‡ 5·2 per cent present as β-glycyrrhetic acid.

Biliary excretion. After oral administration of ^{3}H-β-glycyrrhetic acid as a solution in arachis oil at a dose level of 25 mg./kg. to female rats, more than 70 per cent of the dose of radioactivity was excreted in the bile in 2–3 days, 13–20 per cent was found in the faeces and 2–3 per cent in the urine (see Table III). After oral administration of aqueous suspensions (25 mg./kg.) to male rats an average of 53 per cent of the dose of radioactivity was excreted in the bile in 3 days, a further 11 per cent was excreted in the faeces and 2 per cent in the urine. Female rats in the same time excreted 54 per cent in the bile, 15 per cent in the faeces and 9 per cent in the urine.

The biliary excretion was much greater and much more rapid after intraperitoneal administration of a solution of ^{3}H-β-glycyrrhetic acid in propylene glycol (see Fig. 1). At a dose level of 25 mg./kg., both male and female rats excreted an average of 100 per cent of the administered radioactivity within 12 hr. In fact, 95 per cent of the dose was excreted within 6 to 8 hr. in both sexes. The addition of 0·04 per cent w/v of ox bile salt to the liquid diet did not appear to alter significantly the total

biliary excretion or absorption of the triterpenoid, although in some experiments the initial rate of biliary excretion is slightly lower when bile salt is present in the diet.

Biliary metabolites of β-glycyrrhetic acid. The pooled bile obtained from three female rats, each of which had received 4 mg. of ^{3}H-β-glycyrrhetic acid in propylene glycol intraperitoneally, was chromatographed on Whatman No. 1 paper in solvent A. Two radioactive areas were detected with R_F values of 0–0·1 and 0·15–0·30.

TABLE III

FATE OF ^{3}H-β-GLYCYRRHETIC ACID IN RATS AFTER BILIARY CANNULATION

Expt. No.	Sex	Duration of expt. (days)	Dose per cent of radioactivity found in			Alimentary canal and contents	Total accounted for
			Faeces	Bile	Urine		
Oral doses of 25 mg./kg. in arachis oil:							
9	F	1·5*	7	36	2·4	—	46*
10	F	2	13	72	2·7	—	88
11	F	3	20	73	1·6	—	95
Oral doses of 25 mg./kg. as aqueous suspension:							
12†	F	2·5	14	56	6·6	1·1	77
13	F	3	17	51	12	0·3	81
14†	F	3	31	27	1·1	—	60**
15	M	3	5	55	1·6	0·3	62
16†	M	3	17	56	—	—	73
17†	M	3	—	48	—	—	48
Intraperitoneal doses of 25 mg./kg. in propylene glycol:							
18	F	2	—	93	—	—	93
19	F	2	—	102	—	—	102
20	F	2	—	105	—	—	105
21	M	2	—	102	—	—	102
22	M	2	—	83	—	—	83
23†	M	2	—	109	—	—	109

* Animal died before completion of expt.
** Flow rate of bile impeded. 0·6 per cent in the liver
† Bile salt included in diet.

The material of R_F 0–0·1 was eluted with a mixture of methanol-chloroform (9 : 1 by volume) and the eluate was found to contain radioactivity equivalent to 4·5 mg. of the administered ^{3}H-β-glycyrrhetic acid or to 38 per cent of the dose. Further chromatography of this material on Whatman 3 MM paper in solvent B by descending chromatography revealed two ultra-violet-absorbent, radioactive spots with R_F values of 0·40 (metabolite I) and 0·46 (metabolite II). These two substances were separated by allowing the solvent to run off the front of the paper, the chromatograms being run for a period of 31 hr. The two spots were cut out, eluted with methanol : chloroform (9 : 1 by volume) and the solvent evaporated to give a semi-crystalline residue in each case (24·6 mg. containing metabolite I and 27·9 mg. containing metabolite II).

The material of R_F 0·15–0·30 in solvent A was similarly eluted with a mixture of methanol : chloroform (1 : 1) and the eluate was found to contain radioactivity equivalent to 4·3 mg. of the administered ^{3}H-β-glycyrrhetic acid or to 36 per cent of the dose. Evaporation of the solvent gave a semi-crystalline residue containing metabolite III (32·2 mg.).

The material containing metabolite I, but neither of the other two metabolite fractions, gave a strongly positive naphthoresorcinol reaction for glucuronic acid.

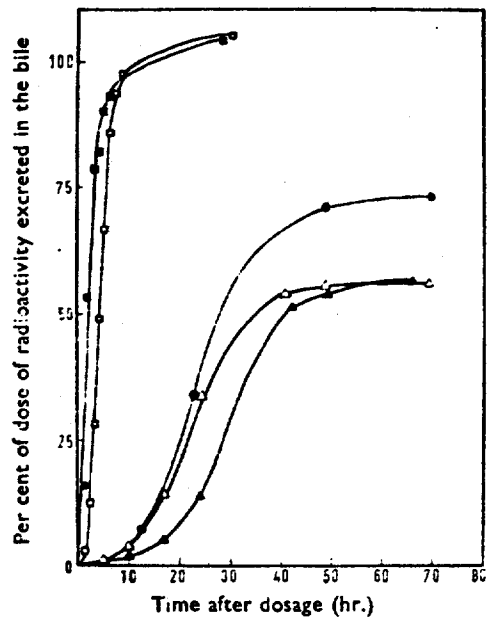


FIG. 1. The rate of biliary excretion of radioactivity following administration of tritium labelled β-glycyrrhetic acid, intraperitoneally in propylene glycol to male rats, □, to female rats, ■; orally as an aqueous suspension to male rats, △, to female rats ▲; and orally as a solution in arachis oil to female rats, ●.

DISCUSSION

Tritium-labelled β-glycyrrhetic acid administered to rats is eliminated from the animal *via* the bile, for almost the whole of an intraperitoneal dose is excreted by this route and only traces are excreted in the urine. The extent and rate of the biliary excretion of this compound is approximately the same in both sexes.

When the compound is administered orally, the biliary excretion is less and the rate of excretion slower than when it is given intraperitoneally. Moreover, the biliary excretion is less and the rate of excretion slower when the oral dose is administered as an aqueous suspension than when it is administered as a solution in arachis oil. Both of these facts suggest that absorption from the gastrointestinal tract is slow, particularly when an aqueous suspension is fed. The replacement of the bile salt lost through cannulation by supplementation with ox bile salt in the diet has no marked effect.

In the previous experiments of Carlat, and others (1959) in which tritium-labelled β-glycyrrhetic acid was fed to man, most of the compound was recovered unchanged from the faeces, only trace amounts were found in the urine, and no activity was detected in the bile. This may have been

because bile was obtained during only the first 4 hr. after dosage, and from our experiments with rats, one would expect the biliary excretion to be about one per cent of the dose, or less, during this period. We likewise found that in the normal rat without biliary fistula, most of the compound was recovered from the faeces, but only a small proportion of this was unchanged β-glycyrrhetic acid. Moreover, since the amount of β-glycyrrhetic acid present in the faeces after oral administration (7·4 per cent) was only slightly greater than that found after injection of the triterpenoid (5·2 per cent), the orally administered compound is probably almost completely absorbed.

The three products excreted in the bile have not as yet been identified but none is identical with unchanged β-glycyrrhetic acid. This may explain why β-glycyrrhetic acid has a healing action for gastric but not duodenal ulcers (see Doll and others, 1962). The β-glycyrrhetic acid being a weak acid, should be largely absorbed from the stomach (Brodie and Hogben, 1957) and although the drug is almost completely excreted again into the duodenum *via* the bile, it is now present only as metabolites which may have no healing activity.

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Two cases of arterial hypertension due to glycyrrhizin intoxication. Analysis of the syndrome by voluntary reintoxication.

by

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Glycyrrhizin intoxication is now well known. Numerous cases have been published in recent years, and since the report of G. Pathé at the Annual Study Group of the French language Society for Nutrition and Diet, even the daily press has dealt with the problem.

Since the first publication from Lyon by Dupré, Favier, Sabourin and Felix, in 1961, Freycon, Froment, Tourniaire, Plauchu have reported some 10 new cases in several publications.

Our own two cases are in no way out of the ordinary, but one of them has made it possible to study the biological disturbances caused by the excessive consumption of glycyrrhizin more precisely, since the patient was willing to undergo a voluntary reintoxication test.

Case 1.

M.S., Antonio, 50 years old, miner, formerly tubercular, severely alcoholic, disintoxicated at his request in July 1967. He had previously been in our service in Feburary 1965 for

asthenia and 11/6 hypotension which suggested Addison's disease, but which was not confirmed by the hormone balance.

He was hospitalized in our service in Nov. 1968 for hemoptysis and the discovery a 25/15.5 hypertension.

Interrogation revealed moderate functional signs: headaches, dizziness and intense asthenia with muscular weakness predominantly in the crural area. There were neither cramps nor paresthesia. Clinical examination revealed nothing out of the ordinary, while ophthalmological examination showed very slight hypertensive retinitis, limited to Gunn's crossing sign.

The electrocardiogram showed a sinus rhythm, elongation of QT and U waves at D 3, V 3, V 4 and V 5.

The ionogram revealed hypokalemic and hypernatremic alkalosis with 79 volume alkaline reserve (35 mEq), 145 natremia, 2.7 kalemia, pH 7.43 and 44 hematocrit.

In view of this pircutre, combining recent arterial hypertension and salt-water disturbances, suggesting a hyperaldosteronism syndrome, questioning was resumed and revealed abundant consumption of beverages with a licorice base since the patient's disintoxication cure, containing roughly 1 g licorice per day.

Examination was then pursued:

The urinary ionogram during a low-sodium diet, 3.5 to 4 g salt (with discontinuation of the toxic substance upon admission), showed very pronounced natruria, of 170 mEq and 27 mEq kaliuria.

Renal function tests proved practically normal aside from an initial albuminuria of 0.22 g per liter, which disappeared in

the following days:

Blood urea 0.30

Addis count 4,400 red cells per minute; leukocytes 50 per min.

Creatinine clearance: 80 ml per min

Urea clearance: 51 ml per min. (Van Slyke: 66%)

P.S.P. 20% at 15 min, 5% at 70 min.

Intravenous urograph: normal.

Calcemia was very slightly lowered, to 94 mg per liter, phosphoremia 22.4 mg per liter, average calciuria 150 mg per day, phosphaturia 1,500 mg per day.

Course

On a low-sodium diet and with no therapy we observed a rapid clinical and biological improvement. Blood pressure dropped progressively until it reached 13 on the 20th day, while all functional signs disappeared within the same period. On the salt-water plane, the alkaline reserve reverted to normal in a remarkable fashion, parallel with the blood pressure, as did natremia, while kalemia rose progressively to 5 mEq (Table 1).

On the urinary plane the massive natruria improved progressively until it reached 60 mEq per day, while the initially very low kaliuria, due to the severe potassium depletion, rose progressively to a normal level of 45 mEq per day.

Reintoxication

The patient was prepared to resume the poisoning under clinical and laboratory surveillance. He ingested 1.5 g glycyrrhizin within a period of 10 days.

We observed a progressive rise in blood pressure which reached 22 on the 10th day, an increase in alkaline reserve, which rose again to 30 mEq, in natremia, which returned to 145 mEq, while kaliuria fell back to 3 mEq.

The urinary changes were extremely typical; intense loss of potassium exceeding 60 mEq per day, while natriuresis fell to about 20 mEq.

Cessation of intoxication was followed by the reverse phenomena. Within 10 days the patient's blood pressure and salt-water balance, both blood and urinary, reverted to normal.

In view of the impossibility of carrying out aldosterone or renin determinations, on the 9th day of voluntary reintoxication we performed a Kaplan test with injection of angiotensin. A positive response of the blood pressure was obtained with a dose of 4 nanogram/kg, which indicates a lower than normal renin activity.

Case II

M.W., Stephane, 57 years old, farm worker, also a former alcoholic who had given up all alcholic drinking for the past 6 or 7 years. Was hospitalized the first time in January 1968 for a kidney colic. Blood pressure was then 19/9.

He was hospitalized in our service in July 1968 for intense asthenia, with headaches, cramps, diffuse pains of recent origin. Blood pressure was found to be 25/13 and a rapid and intense Trousseau sign was noted, as well as a bilateral Chvostek. Questioning revealed that since abandoning alcoholic beverages

the patient ingested fairly large quantities of licorice extract. Summer work on the farm, the high degree of heat during the 15 days preceding hospitalization gave rise to a considerable increase of the ingestion of this substance, corresponding to a value which seemed clearly to exceed 2 g glycyrrhizin per day.

The hypothesis of glycyrrhizin intoxication suggested by the history, and the hyperaldosterone syndrome combining hypertension, extreme asthenia and signs of tetanus, were soon confirmed.

The ionogram showed the existence of hypokalemic alkalosis with a 75 volume alkaline reserve, 1.7 mEq potassium, 144 sodium. Calcemia was slightly reduced, to 80, then 78 and 78 mg/liter. Phosphoremia was 26,then 30 and 30, magnesium 19.6 mg/liter.

In the urine, despite the low-sodium diet initiated upon admission because of the hypertension, natriuria was initially 130 mEq, kaliuria dropped between 15 and 10 mEq per day. There was also a fairly weak calciuria of 160 mg/day.

The electrocardiogram showed a sinus rhythm with a QRS at +30, dome-shaped displacement of ST at D 1, D 2 and right precordial, prolongation of the QT space to 40/100th of a second, distinct U wave at D 2, D 3 and left precordials.

It was noted that renal function was not disturbed: urea 0.30, urea clearance 65.5 ml/min, creatinine celarance 133 ml/min, normal Addis count.

As to the electromyogram, which was only carried out on the 9th day, when the ionogram was almost normal, it only revealed a slight repetitive perischemic activity.

The patient was completely taken off his beverage containing licorice extract from the time of admission. Hypertension and alkalosis improved progressively, but (see Table I) owing to the persistence of hypopotassemia and hypocalcemia, therapeutic intervention became necessary. This explains the somewhat excessive increase in kalemia and the abundance of kaliuria from the 11th day.

It should also be noted that on the 10th day a hypertonic saline serum test (Demanet test) was carried out to investigate the reactivity of the antidiuretic system. This explains the natriuretic peak, but it is also of interest that in this former disintoxicated alcoholic, there was persistent insensitivity of the osmoreceptor centers, which explained the continued polydipsia, with diuresis between 2,500 and 4,000 ml/day during hospitalization.

Discussion

Etiology

Our two patients ingested Antesite, a beverage obtained by dilution of a highly concentrated glycyrrhizin solution. When dilution conforms to the specifications of the manufacturer, it contains 180 to 200 mg glycyrrhizin per liter. It should be noted that other "liquorice beverages" might have similar features, as well as licorice sticks, tablets or squares.

In our two cases we were dealing with two disintoxicated former alcoholics who maintained a certain degree of polydipsia,

and possibly a taste for fairly strong drinks. It should be stressed that both had been drinking Antesite for several years and until the time of their poisoning showed no signs of intolerance. While in the case of the first patient there is nothing to indicate increased ingestion when the poisoning was manifested, a considerable increase in ingestion did take place in the second patient, a farm worker, who, owing to the seasonal nature of his work and the excessive heat, started drinking 5 to 6 liters per day of a more than usually concentrated beverage. He thus undoubtedly consumed more than 2 g glycyrrhizin per day, a dose which is definitely higher than the toxic threshold, which all authors have concordantly set at around 1 g per day.

Symptomatology

The arterial hypertension is the most constant symptom, accompanied by headaches, phosphene, and dizziness. The hypertension may assume all types, but often appears to be fairly severe, giving rise to retinal and renal symptoms and sometimes assuming the course of malignant hypertension. It can cause hemorrhages, particularly retinal or cerebral.

Neuromuscular symptoms, which were very marked in our second case, were only secondary in our first patient. They may be limited to muscular asthenia of varying intensity, but may also give rise to a genuine paralytic picture, and the first case from Lyon (Dupré et al.) was presented as a quadriplegic. They may also include signs of neuromuscular hyperexcitability

with signs of tetanus, and typical alteration of the electromyogram.

Mention should finally be made of electrocardiographic changes: disturbances in repolarization, elongation of QT, presence of U wave.

This entire clinical picture is explained by the laboratory changes, which, when pronounced, give rise to a picture identical to that of Conn's syndrome, of hyperaldosteronism. The blood ionogram shows a combination of metabolic alkalosis, hypopotassemia, hypernatremia, and in the urine a very low natriuria is noted, with a very high kaliuria.

It is interesting that in the majority of published cases the poisoning is stopped by hospitalization, so that the data of the urinary ionograms are very different. From the first days there is a very pronounced sodium crisis, which attenuates as the excess sodium disappears, while kaliuria is initially low, due to the intense potassium depletion.

The voluntary resumption of intoxication clearly shows the effect of glycyrrhizin on the ionic balance and renal elimination (see Table 1): loss of potassium giving rise to a fairly rapid and considerable drop in kaliemia, sodium retention manifested by extreme reduction of natriuresis with elevation of natremia. Together with these two changes the alkaline reserve increases progressively, and the arterial blood pressure rises along a remarkably parallel curve.

These disturbances are quickly reversible upon cessation of poisoning, either spontaneously, or by a brief supply or potassium. In the majority of published cases, as well as in our two cases, the arterial hypertension was also normalized within a period of 10 to 20 days. However, Froment, as well as Plauchu, have reported cases in which the hypertension persisted beyond the discontinuation of poisoning, or indeed existed beforehand. In such cases the glycyrrhizin merely increased the pathological blood pressure values existing beforehand to a varying degree, adding an unusually pronounced sodium retention, not encountered in ordinary arterial hypertension (A. Froment).

As to the physiopathological interpretation of glycyrrhizin intoxication, it remains difficult and controversial. Three preliminary comments should be made:

The clinical picture and laboratory values obtained are identical to those in primary hyperaldosteronism;

glycyrrhizin has long been known to have an adrenocortical stimulating action and Dupré has recalled that its use was formerly recommended in the treatment of adrenal insufficiency;

The toxic effect of glycyrrhizin is experimentally eliminated by total adrenalectomy.

Thus there appears to be agreement that glycyrrhizin gives rise to a genuine primary hyperaldosteronism syndrome by "inappropriate aldosterone hypersecretion" (A. Froment). This concept appears to be supported by certain observations in which the aldosteronuria was found to be very high and renin activity determinations were very low or zero (cases of Tourniaire and

Froment). In our case the Kaplan test with angiotensin carried out on the 9th day of voluntary reintoxication showed a low renin activity. A spirolactone therapeutic test could be added, to correct the ionic disturbances of the syndrome.

Conclusion

Two cases of glycyrrhizin intoxication are reported, involving two former alcoholics, now disintoxicated, but still polydipsic. The syndrome includes constant arterial hypertension, sometimes severe, neuromuscular symptoms with occasional tetanic manifestations and a biological syndrome characterized by metabolic alkalosis with severe hypokalemia and hypernatremia.

The intoxication only develops when ingestion reaches or exceeds 1 g per day glycyrrhizin. Cessation of the toxic substance gives rise to a total regression of the syndrome within some 20 days, but may necessitate added potassium intake.

Voluntary reintoxication showed the speed of action of glycyrrhizin on electrolytes, sodium retention with a drop in natriuresis, potassium depletion by severe urinary loss of potassium, parallel rise in alkaline reserve and blood pressure values.

The syndrome appears to be explained by hyperaldosteronism for which glycyrrhizin is directly responsible, by stimulation of aldosterone secretion.

Tables

1. Case S... 1. Days. 2. Blood pressure. 3. Blood. 4. Re-intoxication. 5. Alkaline reserve.

2. Case W... 1. Days. 2. Blood pressure. 3. Blood. 4. Alkaline reserve.

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Clinical and Pharmacological Investigations on Extract of Licorice

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Extract of licorice never was a matter of any importance in medical science and pharmacology. Although this substance has always been prescribed by physicians in large quantities as a taste-corrector in draughts and as a mass-forming substance in preparing pills, it has never been known that extract of licorice possesses very powerful medical and even toxic properties.

In many countries extract of licorice is used in large quantities especially by children as a pleasant-tasting sweet, but never any unpleasant symptom has been observed. It is very curious that medical science has overlooked till now the extremely remarkable, powerful and even dangerous properties contained in this substance.

Extract of licorice is obtained from the root of the *Glycyrrhiza glabra*. The watery extract from the sweet-tasting root is condensed to a hard consistency. Rods arising in this way can be pulverized into powdered extract of licorice. The plant grows wild and is also cultivated in Spain, Italy, Turkey and Russia.

Some years ago I had some indications that extract of licorice could have a favourable effect on the symptoms of gastric ulcer. For this reason I started experiments in treating gastric ulcer patients systematically with this drug. In the beginning I administered the drug only to patients suffering from gastric ulcer and not duodenal ulcer cases. The reason was that gastric ulcer can be controlled by X-ray examination much better than duodenal ulcers. Later on duodenal ulcers were treated with the drug. The control X-rays were taken once in two or three weeks and the other symptoms, especially pain, were observed at the same time.

All patients had suffered a long time and had been treated before with different methods without success. During the period of administration of extract of licorice no other treatment was given. No diets were prescribed nor rest in bed. The patients were not hospitalized and if possible they continued their ordinary work. In this manner other favourable influences were eliminated.

The preparation and the dose of the drug were the following:

Powdered extract of licorice, 100 g
Water . . . . . 50 g

Three times a day one teaspoon, half an hour before breakfast, half an hour before lunch and before retiring to bed at night.

In this way the patients received the drug on an empty stomach.

The results obtained by this treatment were very remarkable. In a high percentage of cases the X-ray signs of the presence of gastric ulcer diminished in some weeks and even disappeared totally in a short time. Impressive was the disappearance of pain, which happened in many cases after a few days only. The projections gave examples of the X-ray controls and the table gives an insight into the course of 45 cases.

Result of extract of licorice treatment in 45 cases of gastric ulcer.

First control: ulcer disappeared	in 17 cases
First control: ulcer diminished	in 22 cases
First control: ulcer unchanged	in 6 cases
Second control: ulcer disappeared	in 11 cases
Second control: ulcer still present	17 cases
Third control: ulcer disappeared	in 1 case

Afterwards I stated that patients with duodenal ulcers did not react as favourably in such a high percentage as did the cases of gastric ulcer. In my opinion the very intensive anatomical changes with scar tissue in the wall of the duodenum are the reason why duodenal ulcers generally are very resistant to all sorts of treatment.

To get a better insight into the mechanism of the favourable influence of extract of licorice on gastric ulcers pharmacological experiments were necessary. Mrs. and Mr. Nelemans, pharmacologists at the Pharmacological Department of the State University of Utrecht, made for us investigations on the influence of extract of licorice on the intestine of animals. They studied the influence of this drug on intestines suspended in a Tyrode solution and on intestines left in situ. Further the effect of the extract on artificial gastric ulcers in rats was studied by these pharmacologists.

By these experiments the following results were obtained:

1. Extract of licorice reduces the intestinal tonus.
2. It reduces the motility of the intestines.
3. It removes the strong muscular spasm of the intestine induced by acetylcholine, histamine or barium chloride.
4. It can prevent experimental gastric ulcers in rats both in administering it by mouth and parenterally.
5. It has no local anaesthetical effect and no curarine action.

These pharmacological observations gave some support to the observations on patients with gastric ulcers and make it probable that the favourable influence of extract of licorice on gastric ulcers is to be attributed to the removing of spasm conditions from the muscles of the stomach wall. This may cause a better circulation of blood and fluid in the wall of the stomach and can thereby improve the healing of ulcers.

Mrs. and Mr. Nelemans demonstrated further that this spasmolytic power is not connected with the glycyrrhizinic acid that is always present in extract of licorice; the spasmolytic factor is found in the glycyrrhizinic-acid-free part of the extract

and it also seems probable that the favourable effect on gastric ulcers is to be attributed to the same part of the extract.

In the course of my experiments I observed a remarkable and quite unexpected side effect. A relatively large number of patients—about one out of five—showed edema during the treatment with extract of licorice with the above-mentioned dose. This generally begins in the face. The patients look swollen and sometimes they can hardly open their eyelids. In most cases this edema is combined with edema of the legs and hands. Moreover in many cases it is complicated with violent headache, dizziness, oppression in the chest and pain in the upper part of the abdomen. Apparently edema of the brain, lungs and liver is the cause of these very disagreeable symptoms. Complications from the side of heart and kidneys could not be observed, neither could abnormalities of the blood. There was in these cases considerable gain of weight in a few days and a slight hypertension.

The appearance of edema makes it necessary to reduce the dose of extract of licorice or to discontinue the administration. If the daily dose of 10 g (i.e. three teaspoons of the preparation) is reduced to 3 g the edema usually disappears. In some cases however even small doses can cause the formation of edema.

In an attempt to find out which part of the extract had the edema-producing power, I made various experiments. Both boiled and raw extracts caused edema. Extract of licorice from which the proteins had been removed caused edema in a patient with gastric ulcer with a gain of weight of 6.5 kg in a few days. To ten students was given glycyrrhizinic acid, a substance always present in extract of licorice, in a dose of two grams daily. Two of them became edematous in the face and legs, they gained some kilograms in body-weight and they complained of headache.

By these experiments it became evident that extract of licorice contains two substances which are pharmacologically different. One is a substance having a strong spasmolytical power and probably influence on the healing process of gastric ulcers, the other is glycyrrhizinic acid having an edema-producing power.

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p. 1214

Hypertension with primary evidence caused by liquorice

Numerous findings concerning glycyrrhizin intoxication are known; it is a substance inherent in liquorice. As recently recalled by A. FROMENT, H. MILON, J.C. DUPONT, M. PIANTE and R. FROMENT (Lyon medical,1968, 219, No. 22, 1835 to 1858), said intoxication has a rather stereotyped symptom complex, with the following predominant characteristics: it occurs mainly as an after-effect of an alcohol detoxication, after substituting instead of wine,a drink flavored with a liquorice extract (antesite,liquorice water). The revealing symptoms are principally linked to potassium deficiency: neuro-muscular symptoms (asthenia, paralytic seizures and in some cases, tetany). Disorders of the rythm due to vetricular excitability are rarer; potassium deficiency is constant, between 1,6 and 3,0 mEq per liter. The hypertension seems to be likewise constant,although its seriousness varies. As a rule, it remains in the background,but occasionally involves serious symptoms such as retinal hemorrhages. Polydipsia usually occurs, but it is not always possible to regard it as an after-effect of the intoxication, because in numerous cases it is responsible for causing intoxication resulting from an excessive consumption of liquorice. Depending on the seriousness of the potassium deficiency, the clinical aspect according to the above ranges from the paralytic forms to the pseudo-syndrome of Conn. Despite the sometimes dramatic character of the initial symptoms, a cure is possible by suppressing the toxic supply; it has been verified that recovery coincides with a massive sodium depletion and with a considerable potassium retention.

However, a little-known form of the intoxication exists on which present article lays stress for good reasons: it does not involve the evocative potassium deficiency symptoms and the hypertension can be considered primary only when the poisoning is not systematically investigated. Five cases of the above (form) have been observed within 18 months by the cardiology service of the authors.

None of the patients appeared in a particular etiologic context; none had been subjected to an ethyletic detoxication cure and their behavior as well as their activity seemed to be normal. The consultation was motivated by the hypertension only; it had no unusual aspects and had occurred in young men (aged 15 to 45). It was linked to high rates (215-180, 200-100, 170-120, 170-115, 200-110), and affected the eyes to a disquieting extent in three cases (arteriole stricture, edema, retinal hemorrhages or exudation), and had modified the repolarization in 2 cases, but never involved specific symptoms of a renal ailment. The 3 patients affected with a malignant retinopathy had had violent headaches for several months, but none of the 5 had any disabling complication linked to the hypertensive disorder, nor any cardiac insuffiency, nor any vascular brain accident.

Even when oriented a posteriori, the questioning (of the patients) failed to disclose any simptom of potassium deficiency. Only a slight morning asthenia was observed in one patient, and another experienced a slight leg fatigue in the evening only. The fact that these symptoms ceased when the intoxication vanished makes it admissible to connect them with said poisoning. No global asthenia, no paralytic seizure nor any tetanic crises were found. Serous potassium had definitely diminished in 1 case only.

All patients were polydipsic, but the disorder was noticed only after oriented questioning, and the daily diuresis fluctuated between one and three liters.

Recovery followed in all cases after the elimination of the glycyrrhizin; in perspective, after several months without therapy, the blood pressure remained within normal limits.

Accordingly, the hypertension caused by liquorice is very difficult to diagnose because nothing attracts attention; it is also very easily diagnosed with a systematic investigation.

However, it is advisable, whenever a hypertension patient reports on a regular intake of glycyrrhizin, to avoid any a priori statement confirming that said substance is responsible for the hypertension.

In fact, during the 18 months while the preceding cases were under observation, 5 other hypertension patients were observed; they were regular consumers of liquorice; but in case of the latter, the cessation of the intoxication did not produce the expected therapeutic effect abd it had to be admitted that the hypertension was partially or totally unrelated to the same.

Certain basic principles must be taken into consideration.

These 5 patients consumed antecite, while other intoxication cases have been detected after the intake of coconut or of various products based on liquorice. In toxic doses, glycyrrhizin does however cause polydipsia, and the auto-maintainance or the auto-aggravation of the intoxication are definitely promoted whenever the poison is consumed in a liquid form.

Glycyrrhizin seems to have a toxic effect only when the daily ingestion is considerably in excess of the customary consumption. A dosage which is definetly under 0,50 g per day must be regarded as a dose without toxic effect. Only a quotidian consumption results in intoxication. On the other hand, since the effect of the product vanishes within a few days, it should not be held responsible for symptoms which persists for two or three weeks after terminating the consumption of said product.

When hypertension is observed in all members of a family consuming liquorice, the finding speaks in favor of the toxic nature of liquorice.

On the symptomatic level, polydipsia alone is important because it is constant and its aggravation in the course of the intoxication proves the action of the glycyrrhizin. On the other hand, when the neuro-muscular and dyelectrolyte symptoms have a net value which confirme the intoxication, the absence of said symptoms does by no means permit its elimination.

A study of the sodium intake and release balances at the time when the intoxication terminates supplies the decisive facts: the sodium loss is is considerably above its volume observed in primary hypertension patients under identical conditions. This "monstruous" sodium depletion indicates a pre-existent retention, connected with the action of the toxic substance.

Recovery when the intoxication ceases represents the foremost argument; the blood pressure begins to fall around the fourth or sixth day after withdrawal(of the poison), and pressure returns to normal after about twelve days; the retinal lesions and the eventual repolarization disorders likewise improve.

M. KOSTER and G.K. DAVID (New England J. of Med.,1968, 278, No. 25, 1381 - 1382) who recently reported on a comparable case observed at Amsterdam, state that the effect of the liquorice depends on one of its components, the glycyrrhetin acid which-- according to MOLHUSEN-- has effects similar to the effects of the steroids.

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Stimulation of Rat Adrenocortical Cells by Glycyrrhizin with Special Reference to Its Inhibitory Effect on Dexamethasone-induced Atrophy of the Adrenal Cortex

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Adrenocortical stimulation by glycyrrhizin in male rats of Wistar-strain was confirmed by histometrical and enzyme-histochemical methods, and the effect was compared with that of ACTH. Severe cortical atrophy was induced by dexamethasone administration in a dose of 5 mg/kg body weight once daily for 2 weeks. The inhibitory effect of simultaneous administration of glycyrrhizin on the dexamethasone-induced atrophy was less evident than that of ACTH so far as adrenal weight and histometrical observations were concerned. Cortical cell atrophy detectable in animals with 0.25 mg/kg of dexamethasone once daily for 2 weeks was considerably inhibited by simultaneous ACTH administration, and the histochemical activity of glucose-6-phosphate dehydrogenase was reduced. The inhibition was less conspicuous with glycyrrhizin. The histochemical activity of alkaline phosphatase and adenosine triphosphatase in the capillary wall in the fascicular and reticular zones was markedly accelerated with glycyrrhizin and very slightly with ACTH. These histochemical findings essentially conformed to the pattern in the normal animals which were treated with glycyrrhizin or ACTH. It was suggested that the stimulant effect of glycyrrhizin on the adrenocortical cell which resulted in an inhibition of the dexamethasone-induced atrophy is induced through the cortical capillary wall including the adrenal reticuloendothelial cell.

The biologic action of glycyrrhizin on the pituitary-adrenal system would be dual, suppressing and stimulating, according to the dosage and the route of its administration. Atrophy of the adrenal cortex was induced by the treatment with large doses of glycyrrhizin and this effect was interpreted as an expression of the suppressive effect on the pituitary ACTH-release.[1] As to the stimulating effect of glycyrrhizin, simultaneous administration of glycyrrhizin with cortisone had an inhibitory effect on the cortisone-induced atrophy of the adrenal cortex.[2,3] In clinical investigation, administration of glycyrrhizin blocked the effect of dexamethasone in lowering the amount of total urinary 17-OHCS.[4] This finding

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was explained in such a way that glycyrrhizin effectively prevented the suppression of the pituitary-adrenal axis in glucocorticoid induction. This explanation requires further evidence.

The present paper deals with the histopathological and enzyme-histochemical changes in the rat adrenal cortex, which were induced by administration of glycyrrhizin with and without dexamethasone. The results obtained will be compared with those in animals administered with ACTH, and the role played by the cortical capillary wall including reticuloendothelial cells will be discussed.

Materials and Methods

Sixty-seven male rats of Wistar-stain weighing between 130 g and 180 g were used and divided into three groups; 1) 23 normal, 2) 27 treated subcutaneously with 0.25 mg and 3) 17 with 5 mg per kg of body weight of dexamethasone once daily for 2 weeks. The animals of each group were further divided into three subgroups according to simultaneously administered agents: a) glycyrrhizin and b) ACTH-Z, 1 mg/kg body weight subcutaneously once a day, and c) control. The animals were kept on a standard laboratory diet and received daily injections of Achromycin 10 mg/kg body weight. At the completion of the experiments, the animals were sacrificed by ether inhalation 24 hours after the last dose. The adrenal glands were weighed and prepared for further examinations as quickly as possible after sacrifice. The right adrenal gland was used for enzyme histochemistry and the left one was fixed in Zenker-formal for paraffin sections.

Histology and histometry. Ordinary paraffin blocks of the gland were cut into several 4 micron thick sections so as to yield perfect cross sections of the gland including the medulla. The sections were stained with hematoxylin and eosin, Masson's trichrome stain, PAS, or Gomori's silver impregnation. The width of the whole cortex, of the glomerulosa and of the fasciculata-reticularis was measured by a micrometer on the cross section of the gland. The average of three measurements in a section was calculated and evaluated as the index of adrenocortical activity.

Enzyme histochemistry. Materials from the groups 1 and 2 were used for the demonstration of enzymatic activity. Fresh adrenal tissues 3 or 4 in number from animals of each subgroup killed at the same time were quickly embedded in a piece of fresh liver tissue and prepared for further handling in an attempt at easy comparison. Frozen sections 20 micron thick were cut in a cryostat (−18°C) and stained with histochemical methods for 4 hydrolytic enzyme systems and for 12 dehydrogenase and diaphorase systems.

The activities of alkaline phosphatase[5], acid phosphatase,[5] adenosine triphosphatase (ATP-ase),[6] and nonspecific esterase[7] were demonstrated in sections

previously fixed for 3 minutes in cold (4°C) formol calcium solution with sucrose. The staining for succinic dehydrogenase[8] was applied to unfixed sections. Methods for the distribution of glucose-6-phosphate dehydrogenase (G-6-P D),[9] triphosphopyridine nucleotide diaphorase,[9] diphosphopyridine nucleotide diaphorase,[9] DPN-linked α-glycerophosphate dehydrogenase,[10] lactic dehydrogenase,[10] glutamic dehydrogenase,[10] DPN-linked isocitric dehydrogenase,[10] TPN-linked isocitric dehydrogenase,[11] DPN-linked malic dehydrogenase,[10] TPN-linked malic dehydrogenase[11] and β-hydroxybutyric dehydrogenase[11] were applied to sections previously fixed for 5 minutes in cold (4°C) acetone.

Lipid demonstration. Cryostat sections were stained with Sudan black B and oil-red O for demonstrating lipid distribution.

Results

Experiment 1. Normal animals with ACTH or glycyrrhizin administration. The adrenals in the normal controls weighed 27–33 mg on both sides. Histologically, compact cells were predominant in the fascicular zone, and intracellular lipid globules were slightly decreased in general. The findings might be regarded as an effect of Achromycin administration.

In the animals given ACTH or glycyrrhizin alone, no significant changes were observed in the range of adrenal weight and the width of the glomerular and fascicular zones (Figs. 1A and 1B).

Histological responses of the adrenal cortex to ACTH such as lipid reduction and increase of hypertrophic compact cells were particularly evident in the fascicular zone. Less evident lesions were induced with glycyrrhizin; intracellular lipid globules were minute in size without detectable reduction in density. Intracellular colloid droplets were occasionally demonstrated.

In normal animals, alkaline phosphatase was found to be concentrated in the capillary wall, most conspicuously of the glomerulosa, less evidently of the reticularis, and slightly of the fasciculata. In the animals given glycyrrhizin, the enzymatic activity in the capillary wall including the reticuloendothelial cells was markedly accelerated. Less marked changes were induced with ACTH.

The ATPase activity in the rat adrenal gland was distributed predominantly in the capillary wall, and in the normal controls it was moderate in the glomerular zone and the medulla, and to a less extent in the fascicular and reticular zones. In the animals given glycyrrhizin, a significant acceleration of the activity was induced in the capillary wall of the inner cortex. In the animals given ACTH, the distribution of the enzymatic activity remained almost unaltered and conformed to the pattern of normal animals.

Neither ACTH nor glycyrrhizin affected the distribution of acid phosphatase, adenosine triphosphatase, nonspecific esterase, G-6-P D and 11 other dehydrogenase

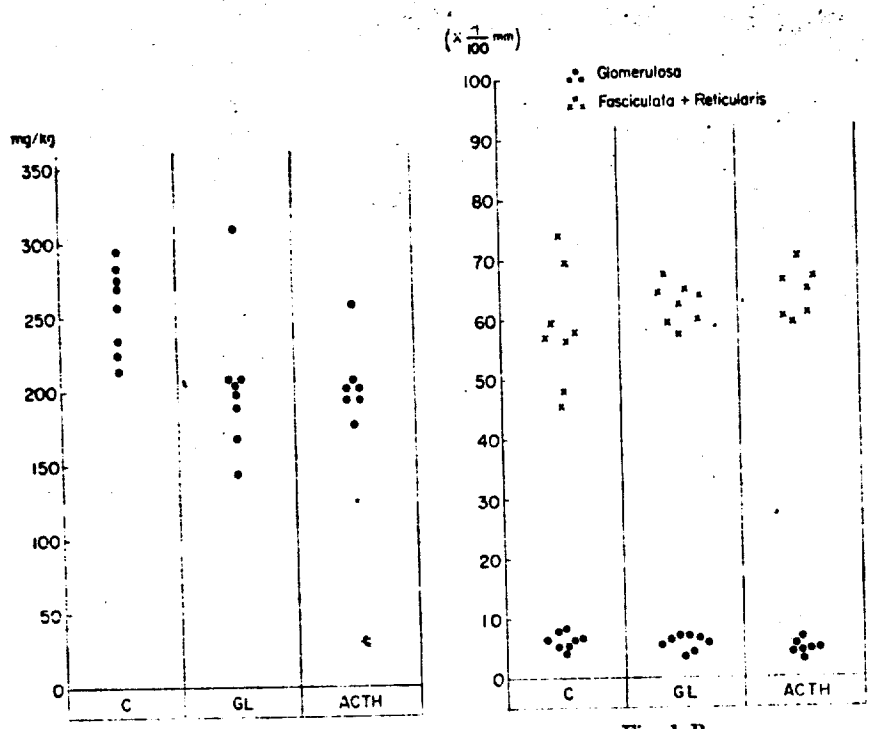

Fig. 1 A. Fig. 1 B.

Fig. 1A. Weight of the adrenal glands of rats in Experiment 1.
C: normal rats. GL: rats treated with glycyrrhizin (1 mg/kg daily). ACTH: rats treated with ACTH (1 mg/kg daily.)

Fig. 1B. Width of the adrenocortical layers in rats in Experiment 1. The codes are the same as those in Fig. 1A.

and diaphorase systems in the adrenal cortex.

Experiment 2. Administration of dexamethasone in 0.25 mg/kg once daily for two weeks. Considerable narrowing of the cortex associated with lipid depletion, an inevitable lesion produced by dexamethasone treatment, was evident in the fascicular and reticular zones. There were neither confirmable lesions of necrosis nor distortion of the cortical cell cord.

The effect of the simultaneous administration of ACTH or glycyrrhizin with dexamethasone was not demonstrated in adrenal weight and in histometrical observation (Figs. 2A and 2B), while it was evident in histologic and histochemical observations.

In the animals treated with dexamethasone plus glycyrrhizin, the histologic lesions of cortical cell atrophy associated with lipid decrease were less evident than with dexamethasone alone. The very slight lesions were observed with

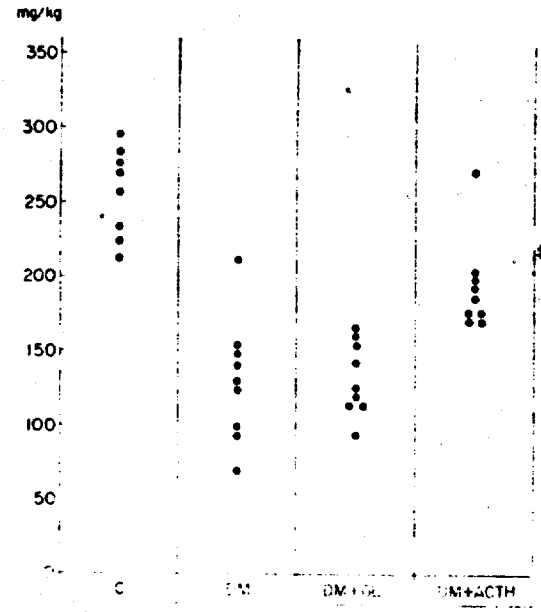

Fig. 2 A.

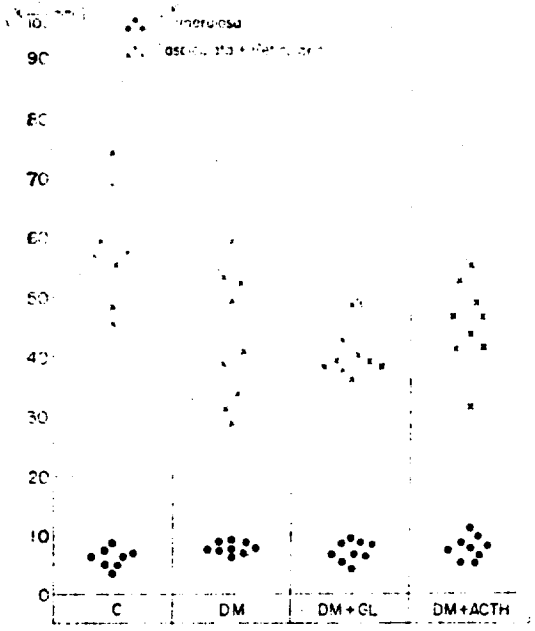

Fig. 2 B.

Fig. 2A. Weight of the adrenal glands of rats in Experiment 2.
Dexamethasone was administered in 0.25 mg/kg body weight daily for two weeks.
C: normal rats. DM: rats treated with dexamethasone.
DM+GL: rats treated with dexamethasone plus glycyrrhizin (1 mg/kg daily)
DM+ACTH: rats treated with dexamethasone plus ACTH

treatment of dexamethasone plus ACTH and there was no difference in the distribution of intracellular lipid globules between the glomerulosa and the fasciculata.

The alkaline phosphatase activity in the capillary wall including reticuloendothelial cells was slightly suppressed by dexamethasone in the fascicular and reticular zones. The suppression was completely abolished by ACTH, and in the animals administered with dexamethasone plus ACTH the distribution of the enzyme activity was slightly more conspicuous than in the normal control. The most distinct acceleration of the activity was observed with dexamethasone plus glycyrrhizin.

Administration of dexamethasone with or without ACTH affected the distribution of the ATP-ase activity in the capillary wall including the reticuloendothelial cells. In the animals given dexamethasone and glycyrrhizin, a considerable increase of the ATP-ase activity in the capillary wall was induced in the fascicular and reticular zones.

In the animals given dexamethasone alone, a slight reduction of the G-6-P D activity in cortical cells was produced in the fascicular and reticular zones. The activity was further suppressed markedly by dexamethasone plus ACTH, and slightly by dexamethasone plus glycyrrhizin.

There were no significant changes in the histochemical pattern of 3 other oxidative enzyme systems and 11 other dehydrogenase and diaphorase systems.

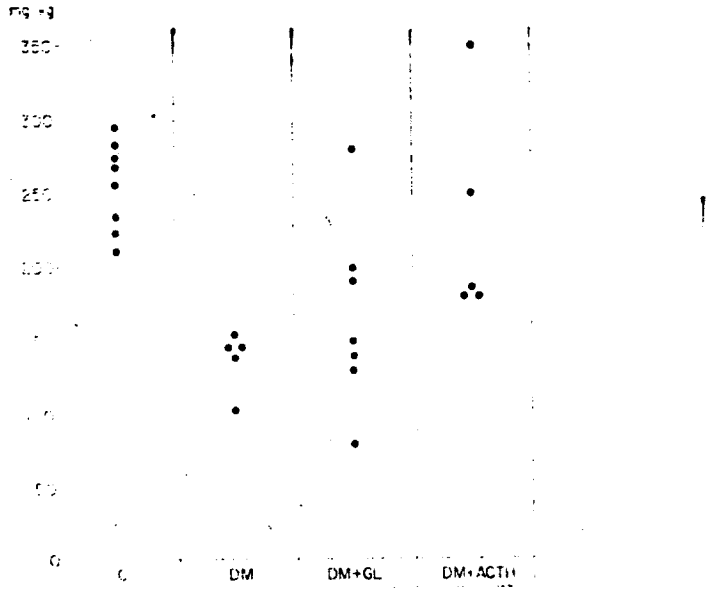

Fig. 3 A.

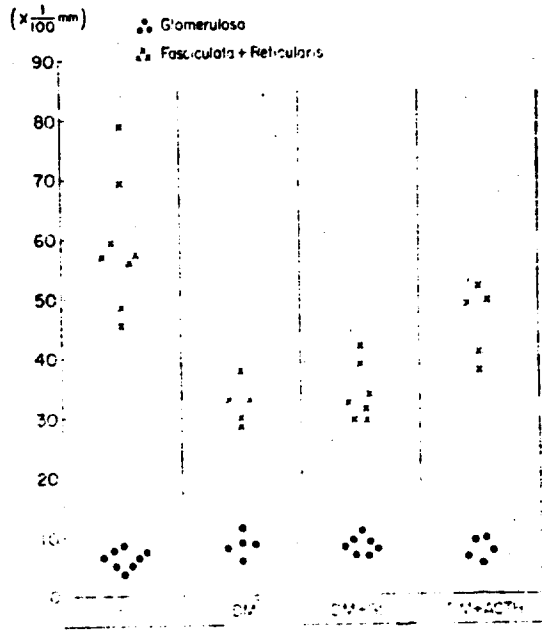

Fig. 3 B.

Fig. 3A. Weight of adrenal glands of rats in Experiment 3. Dexamethasone was given in 5 mg/kg body weight once daily for two weeks. The codes are the same as those in Fig. 2A.

Fig. 3B. Width of the cortical layers in rats in Experiment 3. Dexamethasone was administered in 5 mg/kg body weight once daily for two weeks. The codes are the same as those in Fig. 2A.

Experiment 3. Administration of dexamethasone in 5 mg/kg once daily for two weeks. The inhibitory effect of ACTH on the dexamethasone-induced atrophy was distinct in adrenal weight and histometrical observations. In the group treated with dexamethasone plus glycyrrhizin the inhibitory effect was less evident and observed in about a half of the animals tested (Figs. 3A and 3B).

Histologically, dexamethasone induced severe cortical atrophy with scattered lesions of degeneration and necrosis, and disappearance of cortical cells. The sudanophobe zone was increased in width, and the fascicular zone had an intracellular accumulation of coarse lipid globules resulting in fatty degeneration. Less evident lesions were produced with dexamethasone plus glycyrrhizin. In the animals with dexamethasone and ACTH very slight lesions were observed, and the cortex had the regular arrangement of small compact cells without detectable necrosis. The lipid content was generally reduced, and minute cytoplasmic lipid globules were diffusely distributed without detectable demarcation of the sudanophobe zone.

Discussion

A large dose of dexamethasone, 5 mg/kg body weight once daily for 2 weeks, induced severe cortical atrophy associated with necrosis and fatty degeneration. The inhibitory effect of ACTH on the dexamethasone-induced atrophy with lesions of necrosis and with disappearance of cortical cells, appeared evidently in adrenal weight and histometrical observation. Less evident effect was observed with glycyrrhizin.

In the contrast, in group, which had received dexamethasone 0.25 mg/kg once daily for 2 weeks, cortical atrophy was not advanced. There were no lesions of necrosis and disappearance of cortical cells which were detected in 3 weeks in the previous study[12] of our laboratory. Therefore, the effect of ACTH or glycyrrhizin in preventing the severe cortical lesion did not occur in the present case, and consequently no significant changes were observed in adrenal weight and cortical width. Precise histological and histochemical examinations revealed, however, an inhibition of the dexamethasone-induced atrophy and a reduction of the G-6-P D activity in the fascicular and reticular zones. The findings were evident with ACTH and less conspicuous with glycyrrhizin at identical dosage.

It has been described by several investigators (Kiyono,[13] Aschoff,[14] Jaffe,[15] Akazaki[16]) from the histological viewpoint, that the capillary wall of the adrenal gland contains reticuloendothelial cells comparable to Kupffer's cells in the liver. Local proliferation of reticuloendothelial cells, as well as of lymphatic and myeloid elements in the adrenal gland, may play a role in protecting this important gland against local damages (Selye and Stone[17]). The phagocytic activity of the reticuloendothelial cells in the adrenal gland is, however, less evident than in the liver (Yagawa[18]). On the other hand, the adrenocortical reticuloendothelial cell possesses a great affinity for cholesterol and cholesterol-ester (Jaffe[15]) and is quantitatively responsible for hydroxylation at carbon-17 of the steroid molecule (Berliner *et al.*[19]).

It would be generally accepted that glycyrrhizin stimulates the activity of reticuloendothelial cells; *e.g.*, Kumagai *et al.*[20] observed an inhibitory effect of this agent on the antigranulomatous action of cortisone. The mechanism of the stimulating effect of glycyrrhizin on the adrenal cortex, however, has not been clarified. The present study revealed that in both normal and dexamethasone groups the histochemical activities of alkaline phosphatase and ATP-ase in the capillary wall including the reticuloendothelial cell were accelerated evidently by glycyrrhizin in the fasciculata and reticularis, while such effect was not noticed with ACTH. This result was significant in considering the mechanism of stimulant effect of glycyrrhizin on the adrenocortical cell.

Alkaline phosphatase and ATP-ase in the adrenocortical reticuloendothelial cell, a component of the cortical capillary wall, would not be responsible for corticosteroid biosynthesis. An evident acceleration in the activity of these

enzyme systems, however, indicates a rise in certain metabolism in the capillary wall which probably plays an important part in corticosteroid secretion.

In conclusion, it is suggested that the stimulant effect of glycyrrhizin on the adrenocortical cell which leads to an inhibition of the dexamethasone-induced atrophy as a result of enzymatic hyperactivity of the capillary wall including the adrenal reticuloendothelial cell.

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Explanations for Figs. 4 - 9

Figs. 4A, 4B and 4C. The distribution of alkaline phosphatase stained by Gomori's method in the adrenal cortex of rats in Experiment 1. The activity of alkaline phosphatase is usually concentrated in the capillary wall in the adrenal cortex of normal rats (Fig. 4A), and it is distinctly accelerated by glycyrrhizin (1 mg/kg daily) particularly in the fasciculata and reticularis (Fig. 4B). A similar but a little weak change is observed with ACTH (1 mg/kg daily) (Fig. 4C). × 120.

Figs. 5A, 5B and 5C. Alkaline phosphatase activity stained by Gomori's method in the adrenal cortex of rats in Experiment 2. The activity of alkaline phosphatase in the capillary wall is mildly suppressed by dexamethasone (0.25 mg/kg daily for two weeks) alone (Fig. 5A). A distinct increase of the activity is observed after simultaneous administration of dexamethasone with glycyrrhizin (1 mg/kg daily) (Fig. 5B). The effect of simultaneous administration of dexamethasone with ACTH (1 mg/kg daily) is less conspicuous (Fig. 5C). × 120.

Figs. 6A and 6B. ATP-ase activity stained by Wachstein's method in the adrenal cortex of rats in Experiment 1. The ATP-ase activity in the capillary wall is undetected in the fasciculata and reticularis, while it is evident in the medulla in a normal control animal (Fig. 6A). In the case given glycyrrhizin, (1 mg/kg daily), the activity is evidently increased in the deeper cortex (Fig. 6B). × 300.

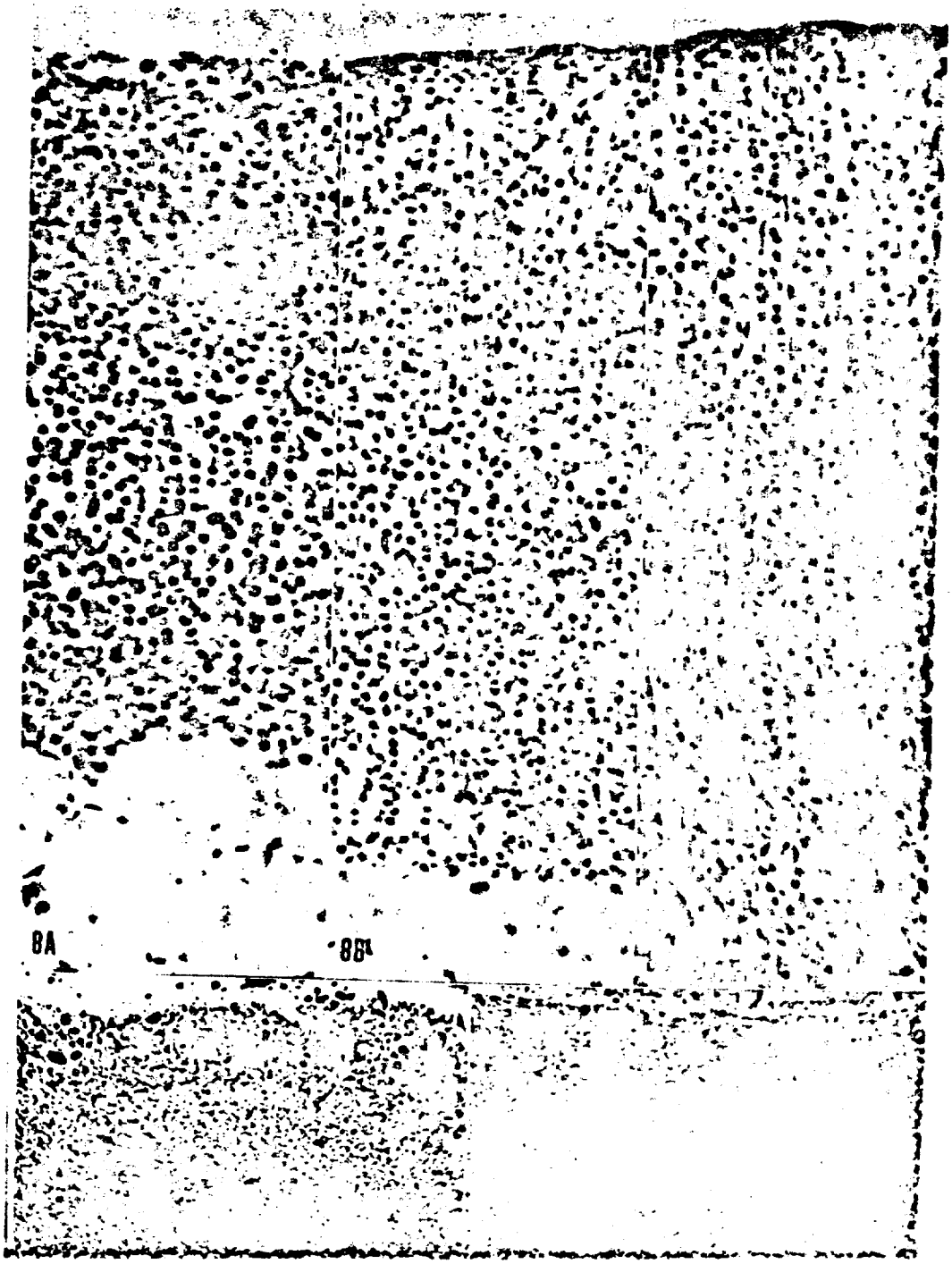
Figs. 7A, 7B and 7C. ATP-ase activity stained by Wachsteins method in the adrenal cortex of rats in Experiment 2. The activity of ATP-ase in the capillary wall is moderately increased through the whole cortex by dexamethasone (0.25 mg/kg daily for two weeks) alone (Fig. 7A). The activity in the fasciculata is significantly increased by the simultaneous administration of dexamethasone with glycyrrhizin (1 mg/kg daily (Fig. 7B), while the effect of simultaneous administration of dexamethasone with ACTH (1 mg/kg daily) is undetected (Fig. 7C). × 120.

Figs. 8A, 8B and 8C. The adrenal cortex stained with hematoxylin and eosin in rats in Experiment 3. Severe degenerative atrophy of the cortical cells was evident through the whole cortex after dexamethasone (5 mg/kg daily for two weeks) alone (Fig. 8A). Cortical atrophy by dexamethasone is considerably inhibited by simultaneous administration of glycyrrhizin (1 mg/kg daily), but mild lesions are still observed in the fasciculata and reticularis (Fig. 8B). The lesions are slight after simultaneous administration of dexamethasone with ACTH (1 mg/kg daily) (Fig. 8C). × 120.

Figs. 9A and 9B. Distribution of lipid stained with sudan black B in the adrenal cortex of rats in Experiment 3. With dexamethasone (5 mg/kg daily for two weeks) alone, accumulation of large lipid globules in the glomerulosa and fasciculata suggests fatty degeneration of cortical cells. The width of the sudanophobe zone is evidently increased (Fig. 9A). Intraplasmic lipid globules, small in size, are distributed extensively in the fasciculata after simultaneous administration of dexamethasone with glycyrrhizin (1 mg/kg daily) (Fig. 9B). × 60.

4A
4B
4C

6B


BA
BB

J. Pharm. Soc. Japan

Takagi, K.[1a], and M. Harada[1b]: PHARMACOLOGICAL STUDIES ON HERB PEONY ROOT. I. CENTRAL EFFECTS OF PAEONIFLORIN AND COMBINED EFFECTS WITH LICORICE COMPONENT F_M100. Yakugaku Zasshi, Vol. 89, pp. 879-886, 1969.

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SUMMARY

The pharmacological studies of central effects of herb paeony root, chiefly of its component paeoniflorin(Pae), were carried out. Herb paeony root is frequently used in chinese medicine. As licorice is one of the crude drugs which are frequently used in combination with paeony, combined effects of Pae with licorice component FM 100 were examined in order to investigate the combined effects of both crude drugs.

Mice were used in these tests. The pharmacological effects of Pae are given as follows. The acute toxicity was very low. Sedative symptom was found. Loss of righting reflex was obtained in the rat by intraventricular administration. Sleeping duration induced by hexobarbital was prolonged. Writhing symptom induced by intraperitoneal administration of AcOH was inhibited. Weak hypothermia and weak anticonvulsive effect to pentylenetetrazol were found.

The pharmacological effects of FM 100 are given as follows. Analgesia was found in both writhing test and tail-pressure test. This action was also obtained in the rat by intraventricular administration in the method which consists in pressing base of the fingers of the hind feet. Marked hypothermic and antipyretic effects and weak anticonvulsive effect to pentylenetetrazol were found by intraperitoneal administration. The combined effects of Pae with FM100 were shown as synergistic in almost all experiments.

Belonging to Paeonia albiflora Pall. var. trichocarpa Bunge, peony is a perennial herb highly popular in Japan for its ornamental and pharmacological values. Its cultivation is concentrated in the prefectures of Nara, Nagano, and Chiba, and its root is utilized as a crude drug [2]. Valued as a medicine for many centuries, it still plays an important role in Chinese medicine and is an indispensable family remedy. The efficacy of peony root is known to be antispasmodic, analgesic, sedative, and astringent, and Shinnohon Sokyo, an ancient Chinese medical record dated between 25 and 220 includes a specific reference to its pharmacological actions (Note 1). Yakucho, written by Todo Yoshieki,

a physician in the Edo era, in 1771 provides a systematic analysis of the medical practice by a school following the theory of Shokanron, also written in ancient China between 25 and 220, and specifies the applications of peony root (Note 2). In Europe, the root of Paeonia officianalis L. belonging to the same genus has been widely used as a folk medicine for the treatment of epilepsy, gynecological diseases, hemicrania, jaundice, hemorrhoids, arthritis, gout, asthma, and other diseases [3].

In pharmaceutical prescription in Chinese orthodox medicine, licorice is often used in combination with peony root. This is a perennial herb belonging to Glycyrrhiza of Leguminosae. There are a number of species, including G. glabra L., G. glabra L. var. Glandulifera Regel et Herder, G. echinata L., G. uralensis Fisch. et DC, etc., and their root and stolon are utilized as crude drugs. They may also be classified as Spanish licorice, Russian licorice, Chinese licorice, and Manchurian licorice, etc., according to the place of production [2]. This herb, used widely in Asia and Europe for many centuries and most frequently in the drug prescription in Chinese orthodox medicine, has a wide variety of application, e.g., as sweetener, flavoring agent, demulcent, and diluent for drug manufacturing. Its efficacy is described in Shinnohon Sokyo (Note 3) and Yakucho (Note 4).

Efforts have been made to clarify the chemical structure of peony root for many years. Recently, Shibata et al. [4] identified the structure of one glycoside, and named it peoniflorin. This is the only special constituent of the herb that has been identified, and no other constituent of pharcological significance has yet been separated. Pharmacological studies so far published include an experiment by Hosono et al. in which peony root extracts were investigated, and a study

conducted by Akabane et al. [5] comprising basic pharmacological tests on a substance in peony root which displayed glycosidic reaction. According to their report, this substance is of low toxicity, and depresses the blood pressure in rabbit, stimulates the intestinal movement, inhibits the movement of excised intestines of guinea pig, stimulates the movement of excised uterus of mouse at low concentrations and inhibits the same at high concentrations. This substance is assumed to be paeoniflorin. Wenzel et al. [3] also reported their experimental findings on the extract of peony root grown abroad, and observed the stimulation of contraction of excised uterus, inhibition of the movement of excised intestines, and stimulation of the heart of frog. There have been a large number of chemical and pharmacological studies on licorice, and their reports provide information on the anti-inflammatory action and DOC-like action of glycyrrhizin, its sweet constituent, and the anti-spasmodic action on the muscles of the aglycon of flavonoid glycoside licylicin* and chalcone-type isolycylicin*. Since Revers reported [6] that this herb was effective for the treatment of gastric ulcer, efforts have been made to pursue its effect on peptic ulcer. The remedial effect of peony root on peptic ulcer was recently confirmed by Takagi, Ishii et al. [7], who obtained a fraction (F_M 100), different from the already known constituent, which exhibited such action, and reported the experimental results on the new constituent along with its general pharmacological properties. Shibata and Saito [8] subsequently conducted an investigation on this substance from chemical standpoint, and successfully separated three new compounds, and identified their structural configurations. Hosono et al. [9] conducted a pharmacological study of peony-licorice solution, a Chinese prescription comprising of the two herbs, in which they observed hypertonia and increased amplitude of rabbit intestine

due to peony root extract and relaxation of muscles due to licorice extract. They also conducted a human experiment on the effects of peony root, and confirmed primarily its positive action on the sleep in both profoundness and duration.

The authors studied the pharmacological effects of peony root, particularly its constituent paeoniflorin, and confirmed various actions of the constituent. The combined effects of F_M 100, above described, and paeoniflorin were also investigated in view of the frequent practice of combining these two herbs in Chinese prescription. It would be of great significance to clarify whether or not such practice results in compound effects between the constituents and the crude drugs, and to determine the nature of such effects if any. This paper discusses the central effects of the two drugs.

EXPERIMENTAL SECTION

EXPERIMENTAL MATERIALS

The peony root extracts and paeoniflorin (Pae), and F_M 100 were purified at the Faculty of Pharmacognosy and Plant Chemistry, Department of Pharmaceutical Sciences, Tokyo University, and Nippon Kayaku Co., respectively. Pae is readily soluble in water, but since F_M 100 is sparingly soluble, it was neutralized with sodium hydroxide and suspended in physiological saline solution. Pae, found to be slightly acidic, was adjusted to pH 7 at use. Since Pae can be decomposed to desbenzoyl-paeoniflorin (Des-Ba-Pae) and benzoic acid, these substances were employed as comparison materials as required. Suitable standard substances were chosen for various pharmacological tests, and those insoluble in water were suspended in CMC. Physiological saline solution was used as the control in the quantities equivalent to those of the drugs.

EXPERIMENTAL PROCEDURES

1. ACUTE TOXICITY. Pae, F_M 100, and a mixture of the two (Pae 2 : F_M 100 1) were administered to male mice (weighing approximately 20 g) by the intravenous, intraperitoneal, and oral routes, and their LD_{50} were obtained based on the mortality rate 48 hours later. The calculation of LD_{50} followed Litchfield-Wilcoxon's method for the intraperitoneal injection of F_M 100 and the mixture solution, and the up-and-down method for other tests.

2. INTRAVENTRICULAR INJECTION OF DRUGS. The procedure followed the method proposed by Takagi and Watanabe [10]. Female white rats (weighing approximately 280 g) were anesthetized with sodium pentobarbital and ether, and their heads fixed. A capped injection needle was implanted from a position 3 mm to the rear and 1 mm to the side of the intersection of the coronal sature and the sagittal sature in a manner that the tip of the needle reached inside the paracele, and fixed. The drug was injected through the needle five days later at a total dose of 0.025 ml, and the animal was observed. The analgesic effect of the drugs was examined by the pressure method [11] comprising the application of pressure at the bases of 3 middle toes of the hind leg. Des-Bz-Pae was employed as the comparison material.

3. EFFECTS ON HEXOBARBITAL-INDUCED SLEEP. The effects of the drugs on the duration of the sleep induced by the intraperitoneal injection of sodium hexobarbital to male mice at 40 mg/kg were studied. The number of animals which lost righting reflex over 2 min and the duration of the loss of righting reflex were examined. The drugs were injected intraperitoneally one hour before the test.

4. ANALGESIC ACTION. (a) Pressure Method. Male mice were subjected to the test, conducted as described in the previous section. The threshold

value of nociceptic reflex at the tail was obtained 8 times at 15 and 30 minutes before the treatment, and 15, 30, 45, 60, 75, and 90 minutes after the treatment. The drugs were applied both orally and intraperitoneally. The maximum threshold values at 15, 30, 45, and 60 minutes after the treatment were obtained to compare the effects of the drugs.

(b) Acetic Acid Method. Whittle's method was employed [12]. Male mice were treated with 0.7% AcOH injected intraperitoneally at 10 ml/kg, and writhing motions were counted from 2 to 12 minutes after the administration of AcOH. The drugs were applied intraperitoneally and orally 30 minutes before the treatment with AcOH.

5. HYPOTHERMIC ACTION. Male mice with rectal temperatures within a specific range were employed, and temperature was taken with a thermistor thermometer at 30 - 60 minutes intervals. This test was performed under the environmental conditions of 22°C and 60% humidity, and each cage contained 10 animals. The drugs were administered intraperitoneally and orally.

6. ANTIPYRETIC ACTION. Intraperitoneal injection of mixed JP VII typhoid-paratyphoid vaccine at 0.5 ml/kg/10 ml of saline produced fever in male mice 2 to 3 hours later. The antipyretic effects of these drugs on the fever were examined. The drugs were so administered that the initial occurrence of fever to a significant degree coincided with the time at which body temperature is depressed by the hypothermic effect of the drugs, described in the previous section, at the maximum rate. In this test, the drugs were injected intraperitoneally 2 hours after the administration of the vaccine, and the rectal temperatures 1 hour later were compared. At this dosage of vaccine, the rectal temperature returned to normal within 24 hours, and no apparent change was noted. In some animals, body

temperature even dropped in 1 to 2 hours after the administration of vaccine, but the animals which exhibited temperature changes beyond a specific range were excluded from the test results.

7. ANTISPASMODIC ACTION. (a) Maximum Electroshock Method. With Woodbury-Davenport's equipment [13], electroshock of 20 - 30 mA was applied for 0.17 sec to both eyes of male mice to which physiological saline solution had been applied. The incidence of tonic extension and mortality was studied. The drugs were administered intraperitoneally 30 minutes before the shock treatment, and orally for 4 consecutive days. In the latter method, the test was performed 30 minutes after the final administration.

(b) Pentylenetetrazol Method. Two methods were employed for the induction of convulsion. The first method comprised the hypodermic injection of pentylenetetrazol at 100 ml/kg 30 minutes after the intraperitoneal injection of the drug, and the examination of the incidence of clonic spasm up to 9.5 minutes later and the number of deaths up to 29.5 minutes later. The deaths were due to suffocation without tonic extension in all cases. The second method followed the procedure of Bastian's experiment [14]: a 1% aqueous physiological saline solution of pentylenetetrazol was administered by infusion from the caudal vein of male mice at 10 ml/hr, 30 minutes after the intraperitoneal injection of the drug, and the time up to tonix flexion or death was measured.

EXPERIMENTAL RESULTS

1. ACUTE TOXICITY. The results are shown in Table1. The intravenous and intraperitoneal injections of Pae caused sedation and hypomotility in the animals. Increased doses resulted in the acceleration of respiration, lack of movement, and occasional clonic spasms. A slight tendency

of sedation was noted even after the administration of F_M 100, and increased doses of the drug produced mild clonic spasms.

TABLE I. Acute Toxicity of Paeoniflorin, F_M 100 and Their Combination

Compound	Route	No. of animals	LD_{50} (mg/kg)
Paeoniflorin	*i.v.*	10	3530
Paeoniflorin	*i.p.*	10	9530
F_M 100	*i.v.*	10	251
F_M 100	*i.p.*	60	641 (571—721)[a]
Paeoniflorin F_M 100	*i.v.*	10	474 237
Paeoniflorin F_M 100	*i.p.*	60	1040 (952—1138)[a] 520 (476—569)

up-and-down method
a) Litchfield-Wilcoxon method
Numbers in parentheses indicate the 95% fiducial limits.

No death was caused within 24 hours from the administration of Pae, but deaths continued to occur even after 24 hours in the group treated with F_M100. The oral administration of Pae and F_M 100 revealed no toxicity within the range which was technically possible. Synergic action was shown by Pae and F_M 100 to some degree, but it was not notable in the intravenous administration. Unlike Pae, peony extract exhibited toxicity, causing deaths following its intraperitoneal administration at 5 g/kg. (Mortality: water extract, 2/5; methanol extract, 4/5).

2. GENERAL SYMPTOMS FOLLOWING INTRAVENTRICULAR ADMINISTRATION OF THE DRUGS. No abnormality appeared following the injection of Pae at 500 ug/animal, but slight sedation and atoxia followed the treatment at 1 mg/rat. The sedation caused by Pae at 5 - 10 mg/rat subsequently developed into sleep with increasing profoundness, followed by bradypnea, loss of eyelid closure reflex, and muscular relaxation. This condition continued for one to several hours, gradually returning to normal in 24 hours. Prior to this condition, some animals exhibited a transient excitation state consisting of spasms, opisthotonum upon touching, biting, etc., immediately after the injection of the drug at a large dosage. No analgesic

effect was noted during the state of sedation. Des-Bz-Pae at 5 mg/rat caused no change in comparison with the control. F_M 100 at 50 ug/rat produced slight sedation, but an increase in dosage, to 100 ug/rat, resulted in slight sedation at first, then cursive spasms in 2 out of 4 animals several minutes later, followed by the acceleration of respiration, piloerection, and crying upon touching, which continued intermittently. One animal died 45 minutes later. Strong analgesic action was observed at this dosage. At 500 ug/rat, the convulsion increased severity, and death occurred sooner. The combined administration of Pae at 1 mg and F_M 100 at 50 ug resulted slight sedation and anaxia, but these symptoms recovered in a short period of time. No increase in analgesic action was noted at this dosage. The results are illustration in Figure 1.

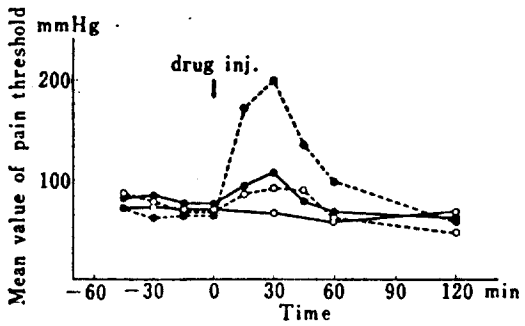

Fig. 1. Effects of Intraventricular Injection of Paconiflorin, F_M 100 and Their Combination on Pain Threshold of the Rat

—○: paconiflorin 1 mg/rat (n=6)
○·····○: F_M 100 50 μg/rat (n=2)
●·····●: F_M 100 100 μg/rat (n=3)
●—●: paconiflorin 1 mg/rat, F_M 100 50 μg/rat (n=3)

3. EFFECTS ON HEXOBARBITAL-INDUCED SLEEP. An example of the results is shown in Table II. In this case, the sleep could not be prolonged by Pae to any significant degree, but other cases showed significant prolongation effects ($p < 0.05$) at a dosage of 1 g/kg (i.p.), the time of sleep being 29.3 ± 6.2 min (n = 8) as compared to the 10.2 ± 4.3 min (n = 7) of the control. This dosage is considered to be the lower limit

that could produce its prolongation effect of any significant degree. In the tests comprising the oral administration of Pae at 1 g/kg and intraperitoneal administration at 0.25 g/kg daily for 4 consecutive days, such effect could not be observed. F_M 100 failed to prolong the sleep, but it exhibited synergic action with Pae. The water extract and methanol extract of peony root administered intraperitoneally gave the effects similar to that of Pae, but the former exerted no influence on the sleep at 2 g/kg of oral administration.

TABLE II. Effects of Paeoniflorin, F_M 100 and Their Combination on Duration of Anesthesia induced by Sodium Hexobarbital on the Mouse

Compound	Dose (*i.p.*) (g/kg)	No. of animals	No. of anesth. animals	Anesthesia time (min)
Control	—	10	4	5.1±2.3[a]
Paeoniflorin	1.0	10	8	9.5±1.8
F_M 100	0.5	10	4	5.2±2.3
Paeoniflorin F_M 100	1.0 0.5	10	8	12.5±2.6[b]

a) mean value ± standard error *b*) significant: $p<0.05$

4. ANALGESIC EFFECTS. (a) Pressure Method. The results of the intraperitoneal administration of the drugs are shown in Table III. The maximum effects of the drugs occurred within 60 minutes. Both Pae and F_M 100 at these doses manifested analgesic effects, but not to a significant degree. Synergic actions of these two drugs could be observed. The oral administration of Pae and F_M 100 at 2 g/kg individually or in combination produced no analgesic effects.

(b) Acetic Acid Method. The results are shown in Table IV. The intraperitoneal administration of Pae and F_M 100 showed notable analgesic effects, the effects shown by F_M 100 being considerable. The oral administration resulted in reduced action of the drugs, to the level not significant. The two drugs exhibited synergic action in both oral and intraperitoneal administration. No analgesic action could be observed when peony methanol extract was given by the oral route at 2 g/kg.

TABLE III. Analgesic Effects of Paeoniflorin, F_M 100 and Their Combination on Pressure Pain Threshold of the Mouse Tail

Compound	Dose (*i.p.*) (g/kg)	No. of animals	Maximum pain threshold (mmHg)
Control	—	10	76.8±9.8[a]
Paeoniflorin	0.5	10	85.6±5.9
Paeoniflorin	1.0	10	98.4±8.3
F_M 100	0.25	10	99.4±7.6
Paeoniflorin F_M 100	0.5 0.25	10	107.0±5.1[b]
Aminopyrine	0.1	10	139.8±13.7[c]

a) mean value ± standard error *b*) significant: $p<0.05$ *c*) significant: $p<0.01$

TABLE IV. Analgesic Effects of Paeoniflorin, F_M 100 and Their Combination on Writhing Symptom of the Mice

Compound	Dose (g/kg)	Administration	No. of animals	No. of writhing (for 10 min)
Control	—	*i.p.*	10	17.9±2.1[a]
Paeoniflorin	0.5	*i.p.*	10	15.5±2.5
Paeoniflorin	1.0	*i.p.*	10	6.1±2.3[b]
F_M 100	0.03	*i.p.*	10	11.3±3.5
F_M 100	0.06	*i.p.*	10	4.4±1.9[b]
Paeoniflorin F_M 100	0.5 0.03	*i.p.*	10	1.3±0.8[b]
Aminopyrine	0.05	*i.p.*	10	11.2±3.2
Aminopyrine	0.1	*i.p.*	10	2.7±1.6[b]
Control	—	*p.o.*	15	26.4±2.5
Paeoniflorin	2.0	*p.o.*	10	18.5±3.1
F_M 100	2.0	*p.o.*	10	19.3±3.2
Paeoniflorin F_M 100	2.0 2.0	*p.o.*	10	13.4±3.0[b]
Aminopyrine	0.1	*p.o.*	5	3.8±3.1[b]

a) mean value ± standard error *b*) significant: $p<0.01$

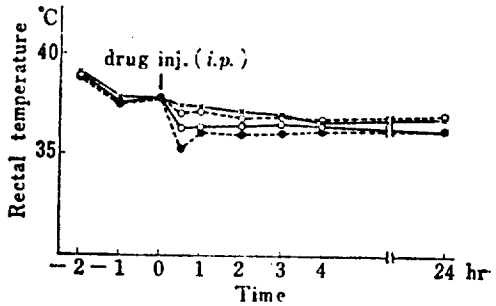

Fig. 2. Time Course of Hypothermic Effects of Paeoniflorin, F_M 100 and Their Combination on the Mouse

×——×: saline 10 ml/kg (*n*=10)
○——○: paeoniflorin 1.0 g/kg (*n*=10)
○——○: F_M 100 0.5 g/kg (*n*=10)
●——●: paeoniflorin 1.0 g/kg, F_M 100 0.5 g/kg (*n*=10)

5. HYPOTHERMIC ACTION. Figure 2 shows body temperature curves resulted from the intraperitoneal administration of the drugs. Changes in body temperature 30 minutes after the treatment are illustrated in Table 4. The rectal temperatures prior to the administration of drug

were within a range of 36.9° - 38.5°C. In this experiment, normal body temperature became lower with time, the lowest rectal temperature occurring 30 minutes after the administration of drug. Pae resulted in a slight drop in temperature, when administered intraperitoneally, and the temperature returned to normal within one hour. FM 100 exhibited strong hypothermic action which lasted considerably longer, over 4 hours. Aminopyrine depressed body temperature for 1.5 hours or so, after which the normal level of temperature could be attained. The two drugs exhibited synergic action to a notable degree. Oral administration of the drugs caused no significant decrease in body temperature. Water extract and methanol extract showed significant hypothermic action when administered intraperitoneally at 0.5 g/kg, whereas the oral administration of the extracts failed to show such effect even at 2 g/kg.

5. ANTIPYRETIC ACTION. Figure 3 illustrates an example of the antipyretic effects of the drugs injected intraperitoneally. Table VI gives the rectal temperatures 1 hour after the treatment. In Figure 3, the temperatures at the time of treatment ranged from 35.5 to 38.2°C. Pae exhibited no antipyretic effect, but F_M 100 depressed the fever over 4 hours. Aminopyrine displayed antipyretic action lasting for 1.5 hour. Synergic action was noted between Pae and F_M 100. Oral administration produced no depression of temperature in the preliminary test (Pae, 2 g/kg; F_M 100, 1 g/kg; Pae, 2 g/kg + F_M 100, 1 g/kg).

7. ANTISPASMODIC ACTION. (a) Maximum Electroshock Method. The results of the single administration and consecutive administration of the drugs are shown in Table VII. Neither Pae nor F_M 100 gave any positive result. The number of the intraperitoneally treated animals surviving the test may be an indication of the synergetic action of the drugs, but the degree of such action is not regarded as significant.

TABLE V. Hypothermic Effects of Paconiflorin, F_M 100 and Their Combination on the Mouse

Compound	Dose (g/kg)	Administration	No. of animals	Rectal temperature (°C, 30 min's value)
Control A[a]	—	*i.p.*	10	37.3±0.23
Control B	—	*i.p.*	10	37.5±0.12
Control C	—	*p.o.*	5	36.3±0.43
Paeoniflorin A	0.5	*i.p.*	10	36.9±0.15
Paeoniflorin B	1.0	*i.p.*	10	37.0±0.14[b]
Paeoniflorin C	4.0	*p.o.*	5	36.9±0.21
F_M 100 A	0.125	*i.p.*	5	36.8±0.30
F_M 100 A	0.25	*i.p.*	10	36.3±0.40[b]
F_M 100 B	0.5	*i.p.*	10	36.3±0.23[c]
F_M 100 C	0.8	*p.o.*	10	37.4±0.27
(Paeoniflorin A / F_M 100)	0.5 / 0.25	*i.p.*	10	35.4±0.27[c]
(Paeoniflorin B / F_M 100)	1.0 / 0.5	*i.p.*	10	35.3±0.30[c]
Aminopyrine A	0.025	*i.p.*	5	36.8±0.25
Aminopyrine A	0.05	*i.p.*	10	36.0±0.21[c]

a) A, B, C: same experimental condition, respectively
b) significant: $p<0.05$, c) significant: $p<0.01$

Fig. 3. Time Course of Antipyretic Effects of Paeoniflorin, F_M 100 and Their Combination on the Typhoid Paratyphoid Vaccine-febrile Mouse

×——×: not vaccinated animal (n=10)
×——×: saline 10 ml/kg (n=10)
○——○: paeoniflorin 0.5 g/kg (n=10)
○——○: F_M 100 0.25 g/kg (n=10)
●——●: paeoniflorin 0.5 g/kg, F_M 100 0.25 g/kg (n=10)
●——●: aminopyrine 0.05 g/kg (n=10)

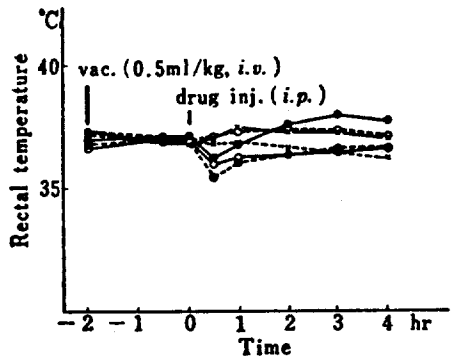

TABLE IX. Inhibitory Effects of Paeoniflorin, F_M 100 and Their Combination on Convulsion and Death induced by Infusion of 1% Pentylenetetrazol in the Mouse

Compound	Dose (*i.p.*) (g/kg)	No. of animals	Time to tonic flexor (sec)	Time to death (sec)
Control	—	10	84± 5	104± 5
Paeoniflorin	1.0	10	87± 8	111± 9
F_M 100	0.5	10	83± 7	115± 7
(Paeoniflorin / F_M 100)	1.0 / 0.5	10	85±12	118±12

pentylenetetrazol: 1% sol., *i.v.* infusion (10 ml/hr)

(b) Pentylenetetrazol Test. The effects of the drugs on the convulsion induced by the subcutaneous administration of pentylenetetrazol are shown in Table VIII, and those in the infusion experiment in Table IX. The antagonistic effects of the drugs against pentylenetetrazol appeared notably in the former experiment, and the prolongation of the time to death was observed even in the infusion experiment. The antispasmodic action

Table VI. Antipyretic Effects of Paeoniflorin, F_M 100 and Their Combination on the Typhoid Paratyphoid Vaccine-febrile Mouse

Compound	Dose (*i.p.*) (g/kg)	No. of animals	Rectal temperature (°C, 1 hr's value)
Control (not vaccinated) A[a]	—	10	36.8±0.20[b,c]
Control A	—	10	37.4±0.15
Control B	—	10	37.4±0.14
Paeoniflorin A	0.5	10	37.3±0.19
Paeoniflorin B	1.0	10	37.5±0.23
F_M 100 A	0.25	10	36.3±0.24[d]
F_M 100 B	0.5	9	35.8±0.30[d]
(Paeoniflorin / F_M 100) A	0.5 / 0.25	10	36.1±0.29[d]
(Paeoniflorin / F_M 100) B	1.0 / 0.5	10	35.7±0.49[d]
Aminopyrine A	0.05	10	36.7±0.29[d]
Aminopyrine B	0.1 (*p.o.*)	10	36.6±0.10[d]

a) A, B: same experimental condition, respectively
b) comparison with the vaccinated control
c) significant: $p<0.05$, *d*) significant: $p<0.01$

Table VII. Inhibitory Effects of Paeoniflorin, F_M 100 and Their Combination on Maximal Electroshock Seizure in the Mouse

Compound	Dose (g/kg)	Administration	No. of animals	No. of tonic extensor	No. of survival
Control	—	*i.p.*	20	19	14
Paeoniflorin	2.0	*i.p.*	10	10	7
F_M 100	0.5	*i.p.*	10	10	6
(Paeoniflorin / F_M 100)	2.0 / 0.5	*i.p.*	10	9	9
Phenobarbital	0.02	*i.p.*	10	0[a]	10
Meprobamate	0.1	*i.p.*	10	2[a]	9
Control	—	*p.o.*, 4 days	9	9	5
Paeoniflorin	0.5	*p.o.*, 4 days	8	8	5
F_M 100	0.5	*p.o.*, 4 days	8	8	4
(Paeoniflorin / F_M 100)	0.5 / 0.5	*p.o.*, 4 days	11	11	6

a) significant: $p<0.01$

Table VIII. Inhibitory Effects of Paeoniflorin, F_M 100 and Their Combination on Pentylenetetrazol-induced Convulsion in the Mouse

Compound	Dose (*i.p.*) (g/kg)	No. of animals	No. of clonic convulsion[a]	No. of death[b]	No. of survival	Mean time to clonic convulsion (min)	Mean time to death (min)
Control	—	18	16	17	0	5.3	17.2
Paeoniflorin	1.0	10	10	10	0	5.1	16.5
Paeoniflorin	2.0	15	13	8[c]	3	>11.5	>32.1
F_M 100	0.5	10	3[c]	5[d]	0	16.1	24.9
(Paeoniflorin / F_M 100)	1.0 / 0.5	10	2[c]	4[c]	0	>27.1	>36.2
Meprobamate	0.1	6	1[c]	0[c]	6[c]	>30.8	—

pentylenetetrazol: 100 mg/kg, *s.c.*
observation time: *a*) 0—9.5 min, *b*) 0—29.5 min
significant: *c*) $p<0.01$, *d*) $p<0.05$

of F_M 100 was stronger, but the effect of Pae could be enhanced by increased dosage, which subsequently prolonged the time to death. Pae prevented some animals from dying, but the survival rate was not significant. The drugs were found to act synergetically. The intraperitoneal injection of Pae at 1 g/kg daily for 3 consecutive days and oral administration of the same at 1 g/kg daily for 7 consecutive days did not produce any effect in the infusion experiment. No appreciable antispasmodic effect resulted from the intraperitoneal administration at 1 - 2 g/kg and oral administration at 4 g/kg.

DISCUSSION AND CONCLUSION

The central pharmacological spectrum of Pae obtained in this experiment will be outlined. The LD_{50} in both intraperitoneal and intravenous administration is extremely large, and the drug seems to be of no toxicity at normal dosage. In a sense, this substantiates the adequacy of its prolonged administration to human in practical application. On the other hand, since the toxicity of a drug can be regarded as indicative of its pharmacological activities, it seems natural that the manifestation of the effects of Pae required relatively large doses. The intraperitoneal administration of the drug was followed by sedation in animal, and this state increased intensity in the intraventricular administration, subsequently developing into sleep with loss of righting reflex. The intraventricular injection of Pae at a dosage of 5 mg/rat normally induces sleep. This is a relatively large dosage, but it still remains within the effective dosage free of toxicity, as indicated by the fact that no death resulted from doses as large as 7 mg/rat and 10 mg/rat. In the F_M 100 experiment which will be discussed in the following section, deaths occurred due to spasms following the intraventricular administration, but

Pae developed no convulsions in the animals. Pae merely increased the depth of sleep with larger dosage, the induced condition subsequently returning to normal. This is of extreme interest. Pae is a glycoside and readily soluble in water. Thus, it is assumed that the penetration of the blood-brain barrier by the substance is difficult in healthy animals.

The drugs which could prolong the sleep induced by hexobarbital do not necessarily possess sedative or sleep-inducing actions, but within the range of this experiment, such actions exhibited by Pae are evidently derived primarily from its sedative effects. The analgesic action of Pae was found to be significant only in the acetic acid test comprising intraperitoneal administration, and its analgesic action is far from being strong. In this method, drugs other than analgesics exhibit some degree of analgesic effects. Thus, the action exhibited by Pae may be of abneural nature. In view of the fact that significant hypothermic action occurred only at 30 minutes after its intraperitoneal administration, Pae is assumed to have extremely weak hypothermic effect. The fever caused by the typhoid-paratyphoid vaccine could not be controlled by Pae. Thus, the central specificity of its hypothermic action seems of no great significance. In addition to the experimental results already discussed, the combined administration of pentylenetetrazol and Pae at a dose 3.3 times larger than that of the former by infusion caused convulsions to a degree comparable to that induced by the injection of the former only. Furthermore, a combination of Meprobamate at 0.1 g/kg and Pae at 1.0 g/kg by the oral route resulted in convulsions intensified synergically. These experimental findings led to an assumption that Pae possessed weak inhibitive action on the pentylenetetrazol-induced convulsions and the transfer of such action to the center was extremely slow and inefficient.

The analgesic and hypothermic actions of F_M 100 have been mentioned in various literatures. The present experiment yielded the following pharmacological spectrum of the drug. The LD_{50} in the intraperitoneal administration was 641 mg/kg, the toxicity being not as low as expected from the results of oral administration. In practical applications, licorice is considered to be free of toxicity when orally taken, permitting its prolonged administration, but its anti-diuretic action following oral administration, which has already been reported, can be regarded as a side effect, and edema and other symptoms have also been noted in clinical cases. The intraperitoneal administration caused sedation in animals, and so did the intraventricular administration. In the latter case, however, the range of dosage inducing this condition was extremely narrow, the condition immediately shifting to death due to convulsion. The fact that death occurs at a low dosage of 100 ug/rat and the dosage also brings about strong analgesic action, and the comparison of the dosage with that for general administration demonstrate that it is also difficult for this substance to penetrate the blood-brain barrier. The analgesic action of F_M 100 was observed in the pressure test and the acetic acid test by the intraperitoneal administration, and even in the intraventricular experiment. This confirmed that F_M 100 definitely possesses some degree of analgesic action. The hypothermic action and the antipyretic action toward the fever produced by a mixed typhoid-paratyphoid vaccine were notable, lasting for a prolonged period following the intraperitoneal injection. On the contrary, the oral administration of the drug was found to be without effect. Practical experience with this drug also denies its possession of anti-pyretic action. It exhibited weak antagonistic action against the convulsions induced by pentylenetetrazol.

The combined effects of Pae and F_M 100 were found to be synergic in

all tests, the synergism being either additive or potentiative. The synergic action in acute toxicity was weak.

The pharmacological effects and the results of the consecutive administration of peony extracts will be discussed in the next report.

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NOTES

* The translator is unable to identify these substances. The Japanese words are transliterated as "RIKIRICHIN" and "ISORIKIRICHIN".

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Pharmacology and Therapy Institute Of the Messina University
Director: G. VINCI

Chemical and pharmacological research concerning the active glycyrrhizin principle of liquorice (Glycyrrhiza glabra L.) typical α glycyrrhizin Reg. and Herd.).

By Luigi Tocco, assistant

(Quotation from Marcus Aurelius, Vol. IV of Reminiscences): Look thoroughly , so as not to miss the value of any subject.

Introduction

Despite the fact that liquorice is a remedy known for a long period of time, our knowledge is still incomplete concerning its composition, its formation and the properties of its active principle, the glycyrrhizin; nor could I find in the bibliography which I was able to scan, evidence of any pharmacological study implemented on the subject.

The studies known to me are limited to the personal research work of WITTE, which indicates a slight laxative and expectorating action, while the effect of the glycyrrhizin and of its salts on the organism is highly important and severe when administered through artificial absorption channels and when prepared with an adequate technical method: the glycyrrhizin is a substance which is difficult to prepare;it is easily alterable. In this comment I refer to the first pharmacological tests and I offer an outline of the preparation methods applied to the substance, reserving the right to report in subsequent comments the results of other pharmacological investigations and chemical tests which I am currently implementing.

General notions and substances used.

The glycyrrhizin, active principle of Glycyrrhiza glabra α typical, is a substance which has not yet been chemically well-defined.

Said name currently refers to a substance which is precipitated from the soaked or infused roots or a decoction of roots, by adding a dissolved acid; the substance has an intensely sweet taste, its reaction is acid. It is obtained amorphous and having a yellowish-brown color by some authors; others obtain it in a white and crystalline

form which exists in the plant according to some AA. , bound to K and Ca, bound to the radical NH^4 according to others, slightly water-soluble, and soluble in alcaline liquids,with a yellowish-golden coloring.

I experimented using either a dry alcoholic extract of a digest at 60^0 of desiccated liquorice root, or I used the glycyrrhizate of ammonium, or glycyrrhizin. The last-mentioned substance is difficult to prepare.

I obtained glycyrrhizin salt with the partial application of methods used by other authors; I modified said methods partially; specifics of the modifications will follow in another report.

From various alcalines, adequate for the preparation of a watery glycyrrhizin solution I preferred, unless using soluble salts, the ammonia and the bicarbonate of sodium; I used them to obtain neutral solutions,or scarcely acid solutions of 4%, occasionally more concentrated, as I will indicate in under various headings. I used frogs, pigeons, guinea-pigs , rabbits and dogs as test animals.

General effect

Frogs. - Glycyrrhizin, administered orally to the frogs is doses ten times larger (1,20-1,40 gr per 100 gr of frog) than the certainly lethal dose injected into the celom, is inactive.

The injection of a lethal 4% glycyrrhizin dose into the celom of a frog was repeated. The animal jumps spontaneously immediately after the injection and its behavior seems normal; but after a few minutes (7 to 10), the frog loses its vivacity, its movements become torpid; it does not react to stimulation and when jumping he withdraws its hind feet slowly, keeping them extended. When placed on its back, the frog attempts to turn with uncoordinated motions, but often fails to do so. Subsequently (25' to 30') spontaneous movements become rarer; the animal remains motionless, the reflexes weaken until, after a certain period of time, they finally disappear to such an extent that the animal does not react when a limb is vigorously pinched or bruised. Repiration weakens, then stops.

when this point of the intoxication was reached I isolated and cut a sciatic nerve between two spines. Electric stimulation of the central(nutilated)stump did not provoke any movement in the rest of the body; when stimulating the peripheral stump I obtained a muscle contraction, showing little enegy,in the corresponding leg; later the muscles failed to react. when exposing the heart it is found dilated, pulsating slowly with weakly. Stimulation with an electric current induced into the venous sinus does no longer cause a heart arrest. When cutting the aorta a little blood drips out at each pulsation; its color is a dark reddish-brown.

The heart stops in diastole.

The lethal dose for frogs, administered by injection, is 12 to 14 cg of glycyrrhizin per 100 g of frog.

This dose,when injected into the lymphatic sacs or into the muscles is,however,not sufficient for killing the frog; given through these paths a larger dose is required (18 to 21 cg per 100 gr of frog) and death occurs 6 to 7 hours later with the above-mentioned symptoms.

When injecting doses much below the lethat volume (6 to 7 cg. per 100 g of frog),either into the celom or into the lymphatic sacs I always observed a great depression, uncoordinated movements; however, the intensity of the above symptoms soon diminished and the animal recovered after 4 to 6 hours.

<u>Pigeons.</u> - The animals recieved subcutaneous injections of glycyrrhizin doses amounting to 0,45 to 0,50 g per kg.;one hour after the administration, abundant excretion of excrements, first solid, then liquid, of yellow color; considerable depression and regurgitation of yellow-colored substances which, when treated with concentrated nitric acid, react like bile pigments.

The depression and the other disorders disappear in about 24 hours.

<u>Guinea pigs</u>. - A glychirrizin dose of one g per kg of animal, injected subcutaneously, kills the guinea pigs in less than 24 hours.

Approximately ½ hour after the injection the animal moans, is torpid and inclined to sleep; a strong general depression follows, the cardiac pulsation number dimnishes, urinary secretion becomes scarce and the excreted feces indicate diarrhoea.

Autopsy result: flaccid heart stopped in diastole; internal organs colored yellowish-brown, bladder empty.

For these tests I used a watery ammonium solution of glychyrrhizin of 7%.

Dogs.-The glychyrrhizin is orally administered; dosage: 1 to 2 g per kg of animal; a few hours later no other symptoms are observed except some alvine discharge, first hard, then slushy; but a depressive condition follows, as in frogs, whenever the substance is administered subcutaneously or endovenously.

Subcutaneous: I have always administered these injections without fastening the dog; it was hled by an assistant, extended on its flank. I injected at various points of the back; this was followed by vigorous massage. During these operations the dogs did not move or complain.

I administered in this way doses of 0.30 to 2 g per kg, dissolved in 50 cm^3 of water distilled with ammonia or bicarbonate of soda until a neutral or slightly alcaline reaction occurred.

As soon as released the dogs appear normal at first; then they become somewhat restless, laying down and rising repeatedly as if they felt uncomfortable in any position; then slight depression symptoms appear which, however, vanish in 1 to 3 hours, even with strong doses. One or two discharged alvines, at first hard, then soft; staggering walk, diminished respiration frequency, sleepiness.

Endovenous: glycyrrhizin administered in this way is lethal in a dose of o,45 to 0,50 per kg. of dog. As the drug slowly flows through the veins the dogs cease to whine and do not struggle any more. Respiration becomes superficial, pulse frequency is reduced.

Some vomiting follows; blood pressure falls and the heartbeat becomes so feeble and rare that when exposed by incision, the carotide discharges ½ to 1 cm^3 of non-coagulating blood only after each pulastion (43 per 1').

When the injection is interrupted at this point, the wound rapidly sutured and the dog released, it is found that sensibility and mobility are thoroughly altered.

The animal is extended on its flank, hardly reacts to pinching or to any other stimulation and is from time to time shaken by convulsions; it discharges at first solid feces, then liquid feces. It remains in said position for a prolonged period of time (6 to 7 hours). Cardiac pulsation becomes slow and extremely weak, respiration becomes stertorous and the animal dies.
I report a test:

Test 1 - Dog, male, of 9.500 kg, fasted for 12 hours.
9 a.m. - Fastened the animal in restraining apparatus; isolated the g.s. vein and inserted an injection cannula in the same, for the endovenous injection with rubber tube, connected with a graded burette containing 4,50 g of glycyrrhizin in 40 cm^3 of precisely alcalized water up to a neutral reaction of $NaHCO^3$.
9.30 a.m. - I let the liquid flow slowly into the vein (1 cm^3 every 30 seconds).
9.35 a.m.- The animal does not complain any more. Repiration is superficial, pulsations frequent and slight.
9.45 a.m. - 30 cm^3 of the liquid was injected. The animal is motionless; conjunctival reflex well-preserved. Heartbeats intermittent and very weak (43 per 1').
9.50 a.m. - After termination of the injection the animal is released. It lies on its flank. Does not react when shaken or pinched.
12 noon. - Lies still motionless. Slight dispnea. Vomiting. When vigorously shaken, slight reaction with some head movement.
4 p.m. - The animal is still motionless. Discharges first solid, then liquid feces. Heart continues to weaken.
5 p.m. - The animal expires without convulsions.

* * *

It results from these tests that the glycyrrhizin is lethal for the currently used test animals when administered through an artificial absorption path; death occurs with symptoms of strong general depression, specifically affecting the heart and the central nervous system.

Effect on the spinal medulla

Glycyrrhizin, when directly introduced into the spinal medulla of frogs as well as dogs with increased dosage, causes at first uncoordinated movements and a slight paresis while sensibility remains sound or is slightly reduced; finally, reflexes and sensibility are completely lost: the administered doses are minor and are such that they would not cause any disorders when administered with endovenous injection.

I report a few tests:

Test II. - Frog, of 35 g.

5 p.m. - Fastened the frog on the small board and exposed the spinal column; expose the medulla to the length of one vertebra. Released, the animal jumps normally.

6 p.m. - I insert into the spinal medulla a drop of the 4% glycyrrhizin solution.

6,5 p.m. - After jumping, the animal withdraws its hind legs slowly.

6,7 p.m. - Jumps with effort and when withdrawing its hind legs the movements lack coordination. Sensibility is retained.

6.10 p.m. - The tensor muscles lose their supremacy over the flexors; the frog bends its limbs with difficulty; after jumping, the limbs remain extended.

6.30 p.m. The frog is motionless with extended legs. Hardly any reaction to stimulation.

7.15 p.m. - Is found dead.

Test III. - Dog, 8 kg.

Tied the animal face downward on the restraining apparatus; introduce a glass syrinx transversally forward into and downward into the lumbar region and penetrate between the last lumbar vertebra and the first sacral.

4 p.m. - Injection of 24 cg (3 cg per kg) of glycyrrhizin distilled in 2 cm^3 of distilled water and precisely neutralized with bicarbonate of soda. The animal does not complain. When releasing it I observe a slight paresis of the hind quarter; when made to run, it crosses its hind legs and falls. Sensibility is retained.
4.10 p.m. - Walks with difficulty and performs uncertain movements with its hind quarter.
6.15 p.m. - Some muscular contraction of a tonic type becomes evident.
4.20 p.m. - The animal is very depressed. Rests on its belly without moving, with its hind legs extended. Intense dispnea. Vomiting. Does not react to pinching.
4.30 p.m. -Loses feces and urine. - Dispnea.
4.45 p.m. - Still motionless with extended limbs. Strong dispnea.
4.55 p.m. - Expires.

* * *

These, as well as numerous other tests which I do not report for the sake of brevity, show that glycyrrhizin when introduced into the spinal medulla or when injected into the rachidean channel in small doses, kills the test animals, causing first paresis and a general sensibility depression, as found by us when investigating the general effect; this is followed by loss of sensibility and complete paraplegia.

Effect on the cerebral cortex

I studied the effect of glycyrrhizin on the cerebral cortex either by applying it directly to the exposed cerebellum, or by submeningeal injection. Any of these testing methods always results in a depressive effect.

When a drop of the glycyrrhizin solution is placed on the exposed cerebellum of a frog, the animal immediately behaves like a brainless frog.

When, after cutting an opening into the cranium of a dog, a small quantity of the glycyrrhizin solution is injected under the meninges, the animal remains motionless as if in deep sleep; sensibility is retained at first, then diminishes and disappears.

Subsequently, some convulsions of the tonic-clonic type occur; the animal vomits, loses feces and expires. Due to local application, the electric excitability of the cortex is also considerably reduced.

I report the protocols a some tests:

Test IV. - Frog of 36 g.

4 p.m. - Is fastened on a small board and I expose the cerebellum. When released, the frog jumps, seemingly normal.

4.20 p.m. - I introduce into the cerebellum a small drop of 4% glycyrrhizin.

4.22 p.m. - The animal is motionless, insensible to its surroundings. It jumps when stimulated. Turned on its back, it vivaciously returns to its normal position.

5 p.m.- The frog still remains in the same position.

6 p.m. - Id. When stimulated, reacts less vivaciously. The test is discontinued.

Test V. - Dog, 5,5000 kg. The animal is tied to the apparatus resting on its belly. I cut a small hole into the cranium and enlarge it to permit the introduction of a syrinx needle under the meninges.

3 p.m. - I inject 9 cg of glycyrrhizin dissolved in 2 cm^3 of distilled water. The animal relaxes immediately.

3,2 p.m. - The dog is motionless as if in deep sleep. It reacts to pinching.

3,7 p.m. - Whines, executes some uncoordinated movements. Minimal reaction when pinched.

3.15 p.m. - Still remains motionless, extended on its flank. From time to time the animal is shaken by convulsions of the tonic-clonic type. Insensible to stimulation.

3.20 p.m. - Loses feces. Dispnea.

3.25 p.m. - Makes some uncoordinated movements and dies.

* * *

I believe that the following conclusion can be reached from these tests and from other tests which I do not report: glycyrrhizin, when applied to the cerebellum in small doses which would be harmless when endovenously injected, causes serious depressive symptoms, abolishes sensitivity to pain and subsequently kills the animals.

Effect on the heart and on the circulation.

In the chapter entitled "General effect" I reported that the substance at issue diminishes cardiac activity and can even paralyze the heart completely; these changes can be followed step by step when exposing the heart of a frog intoxicated with glycyrrhizin.

After a variable period of time following the administration of the drug, within a few minutes when it is injected into the celom, and later when subcutaneously injected into the thick muscles of the tight, it became evident that the heart fills to a lesser extent and the systoles are less vigorous; subsequently, heartbeat frequency diminishes. This becomes increasingly evident; the amplitude of the diastole increases, the pause lengthens. Finally, temporary heart arrests occur with increasing frequency, and the heart stops terminally in diastole.

The animal hardly reacts at all to stimulation; after a while no reaction occurs.

Test VI. - Frog, 30 g - Fastended to a cork, the heart is exposed.

1 Tempo	2 Pulsazioni cardiache in un minuto primo	3 Osservazioni
4 ore 15,45	80	
» 15,50	80	
» 15,55	80	
» 16		5 iniettato gr. 0,04 di glicirizzina in ½ cm³ nel celoma.
» 16,5	80	
» 16,10-16,15	80	
ore 16,20	74	
» 16,25-16,30	60	
» 16,35	50	
» 16,40-16,45	36	
» 16,50-16,55	32	
» 17	30	
» 17,10-17,20	18	
» 17,20-17,25	8	6 Cuore flaccido arrestato in diastole, toccato si contrae appena

1. Time 2. Cardiac pulsations during the first minute
3. Remarks 4. Hours
5. I inject 0,04 of glycyrrhizin in ½ cm³, into the celom
6. Flaccid heart, arrested in diastole. When touched, hardly any contraction.

An identical behavior is shown by the heart of a frog to which a strong dose of atropine (-0,005 g) was adminsitered.

Glycyrrhizin produces a paralysis of the intercardiac arrest system. It is a fact that when the borderline between the venous sinus and the auricle is stimulated with induced electric current in a frog intoxicated with glycyrrhizin, a heart arrest is obtained at first; this will no longer occur when the intoxication has progressed further.

In the dog the endovenously injected glycyrrhizin increases cardiac frequency at first; this is followed by the progressive reduction of the same, until the heart is arrested in diastole. Blood pressure does not increase, but decreases progressively from beginning to end.

Identical results are obtained with animals to which atropine was given.
I report a test:
Test VII - Dog, male, 9,500 kg.
The animal is fixed to the restraining apparatus. R. carotide connected with a mamometer with Hg and recorder on a revolving blackened cylinder. I prepare the v.g.d. for the injection of the poison.
I learned from the layout:

1 Periodi di osservazione	2 Pulsaz. cardiache media per m.	3 Pressione in m/m
9,25-9.30	148	160
9.30-9.40 4 Iniettо gr. 4,50 di glicirizzina in 40 cm³ lentamente (2 cm³ ogni 1')	170	132
9.40-9.45	120	102
9.45-9.50	66	94

1. Observation period.
2. Average cardiac pulsation per m.
3. Pressure in m/m
4. I inject 4,50 of glycyrrhizin in 40 cm³ slowly (2 cm³ every 1')

The animal relaxes and remains motionless on its flank when placed on the floor. The number of the pulsations continues to decrease: 50 pulsations per 1' are counted before the animal expires at 17.

* * *

It can be deduced from the total of the tests which I do not report for the sake of brevity that the glycyrrhizin acts at first as a depressant, then as a paralyzer of the moderator and accelerator systems of the heart and of the myocardium itself.

Initially, the depressive effect on the moderator systems (vagus) predominates and the cardiac frequency increases; subsequently, the depressive effect reaches the acceleration systems as well and then a heartbeat frequency decrease results, which becomes increasingly conspicuous until heart paralysis takes place.

Effect on the sensory and motor nerves

Motor nerves. - When reporting tests concerning the general effect I stated that when the peripherical stump of the sciatic (nerve)was electrically stimulated I obtained, admittedly not very vigorous, contractions of the corresponding leg muscles, which later failed to react. I had therefore to ascertain whether the fact should be attributed to the paralyzing effect of the glycyrrhizin on the nerve, on the intramuscular nerve ends,or on the muscle.

To investigate the question: which anatomical elements are affected by the drug, I made two gastric-sciatic frog (nerve) preparations and placed them, as did BERNARD during the classic tests concerning curare, in two glasses.Into the glass containing the muscle I poured a solution of 4% glycyrrhizin, and into the other which contained the nerve I poured a physiological solution; I proceeded vice versa for the other nerve-muscle preparation.

I accordingly found that the muscle submerged in the glycyrrhizin solution rapidly lost the ability to contract, either when directly stimulated or when the nerve was stimulated; an increasingly strong induced voltage was needed to obtain a reaction; no reaction was obtainable after 30'.

On the other hand, the muscle submerged in the physiological solution maintained the integrity of its excitability. The nerve submerged in the solution of the drug lost its excitability rapidly; an increasingly strong stimulus was needed to obtain a contraction, until after 40' the nerve could no longer be excited, while the muscle retained its full contraction capacity.

I repeated these tests several times with a slightly varied plan, for example: by suspending the nerve-muscle preparations vertically so that in one of them the nerve floated in the solution of the drug, and the muscle (floated) in the other. The results were identical.

* * *

It results from said tests that <u>the effect of the glycyrrhizin on the motor nerves is at first depressive and then paralyzing; it also affects the muscles in this way</u>, as reported at length in the chapter entitled "Effect on the muscles" in another comment which I am currently preparing.

<u>Sensory nerves.</u> - For the implementation of these investigations, I amputated the medulla under the bulb of two large frogs; after 6 hours I separated a sciatic nerve from both which I severed below, jointly with the gastric.

When the central stump was stimulated the frogs raised the opposite lower limb.

I suspended the frogs so that, while they remained facing up, their sciatic nerve, kept extended by the weight of the gastric, floated in a 4% glycyrrhizin solution; the other floated in a physiological solution. I stimulated the nerve electrically at equal intervals and I found that the opposite limb was raised. The nerve submerged in the glycyrrhizin solution rapidly lost ist excitability until approximately after the first 50 (seconds) I no longer obtained any reaction in the opposite limb, while in the other frog the nerve submerged on the physiological solution lost its excitability after approximately 120'.

Effect on the gustative sense

As known, liquorice has the capacity to mask certain nauseating or bitter tastes. Since I am not aware that anyone has supplied numerical test data on the subject at issue, I undertook research related to the same, following the technical method of GARDELLA, limited to bitter taste only(hydrochloride of quinine), which I summarize on the following Table.

1 Individui sesso-età	2 Soglia normale ‰	3 Durata applicazione di sol. al 4 % di glicirizzina	4 Soglia dopo l'applicazione ‰	5 L'amaro diventa nauseante ‰
6 L. T. maschio 30	0,004	2'	1,00	4,50
R. V. 23	0,005	2'	0,90	4
S. G. 42	0,004	2'	0,80	4
7 L. T. s. detto	0,004	5'	1,50	5
R. V. idem	0,005	5'	1,20	4,50
S. G. idem	0,004	5'	1,40	4,75

1. Individuals - sex - age
2. Normal threshold - ‰
3. Duration of the application of the 4% glycyrrhizin solution
4. Threshold after the application - ‰
5. The bitter(taste) becomes nauseating - ‰
6 male
7. ditt.

Therefore the glycyrrhizin acts depressively un the nerves of the gustative sense and on the gustative nerve ends .

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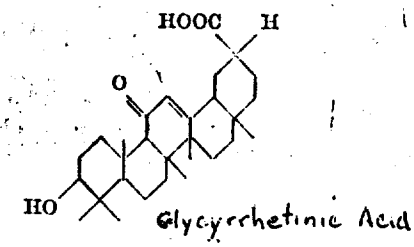
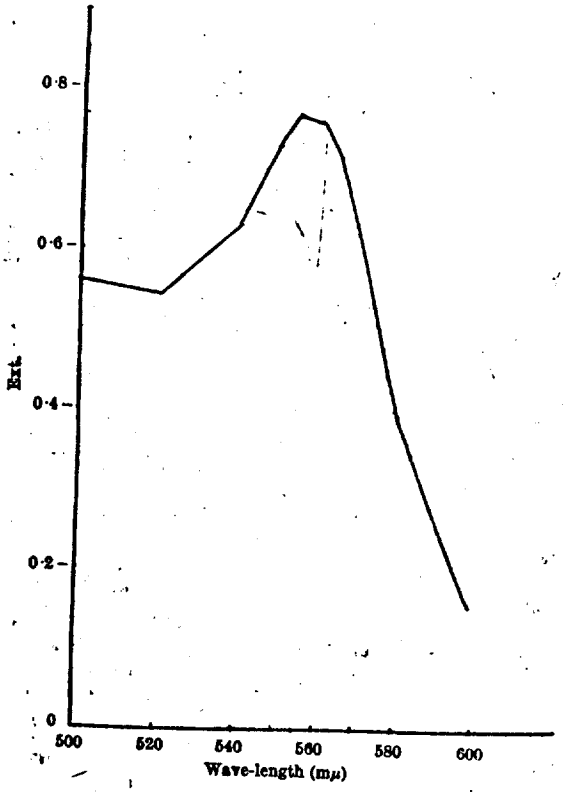
Metabolism of Glycyrrhetinic Acid in Human Subjects

In a series of publications Groen, Pelser, Frenkel, Kamminga and Willebrands[1-6] have reported on the successful treatment of Addison's disease by extract of liquorice, glycyrrhizinic acid or glycyrrhetinic acid. It was shown that either the crude liquorice extract or its active component produced an effect on electrolyte metabolism which was entirely comparable to that of deoxycorticosterone, in a similar way to that already demonstrated by Borst *et al.*[7] in normal individuals.

Following a suggestion made by Groen and his co-workers, we have recently worked out a method by which the urinary excretion of glycyrrhetinic acid in amounts as small as 0·01 mgm. per 24 hr. could be determined without difficulty[8]. Using this method, however, no glycyrrhetinic acid could be detected in the urine of a patient with Addison's disease who was treated with 2·28 gm. of glycyrrhetinic acid per day orally. The urine of a patient with a jejunal ulcer who received 2·5 gm. of glycyrrhetinic acid per 24 hr. by mouth also failed to show a trace of this compound.





Further investigation was therefore directed towards the detection of possible catabolites of glycyrrhetinic acid. It was found that, after administration of glycyrrhetinic acid, extraction of the urine with butanol at room temperature yielded a substance which gave a red colour with 71 per cent sulphuric acid in methanol solution. Up to the present, we have examined a total of twenty urine samples from different patients during periods when no glycyrrhetinic acid was administered and thirty samples of urine from periods during which this compound was given. Of the first-mentioned twenty samples only one gave a positive reaction with sulphuric acid, and this was the urine collected on the first day after the interruption of the treatment. The thirty samples collected during the treatment with glycyrrhetinic acid all gave a positive reaction with the sulphuric acid reagent. The optimal conditions for development of the red colour are being studied. Glycyrrhetinic acid itself does not give this reaction.

The accompanying figure shows the absorption spectrum of the red solution, as measured with the Beckman spectrophotometer. The absorption maximum lies between 555 and 560 mμ.

The investigation will be continued, and an attempt will be made to isolate this glycyrrhetinic acid derivative in the pure state and to identify it. It is hoped that this may throw new light on the mechanism of action of glycyrrhetinic acid on water and electrolyte metabolism in human subjects.

The investigations were carried out in co-operation with the Second Medical Service, Wilhelmina-Gasthuis, Amsterdam.

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Jan. 14.

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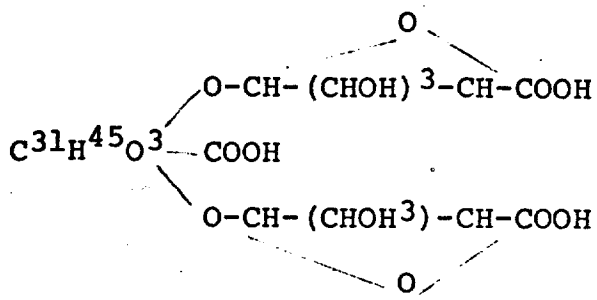
Comp. Rend. soc. Biol.
111: 354-355, 1932

Action of Potassium Glycyrrhizate on Tetanus Toxin

by

Leon Velluz

Glycyrrhizic acid or glycyrrhizin, as is known, is a complex tribasic acid, very poorly soluble in water, with the following formula:



It yields well-crystallized, very readily soluble potassium and ammonium salts that have an intensely sweet taste. To this physiological property which has long been put to use, the following property is added based on my investigations, which has not so far been reported: the soluble salts of glycyrrhizic acid are definitely cryptotoxic for tetanus toxin. This is one of the first examples of a complex molecule which has this unusual property.

Ammonium glycyrrhizate in a 5% quantity is added to a tetanus toxin with a lethality of 1/800 cc. After 4 days of contact at 38-39°, the injection of 1 cc of the mixture is no longer pathogenic for guinea pigs. If the same test is repeated, as was done by this author, with chemically pure tripotassium glycyrrhizate, the same neutralization is observed. It is thus

sufficient to use 5 mg glycyrrhizin in the state of soluble salt to transform 800 lethal doses of tetanus toxin into an inocuous cryptotoxin (1).

(1) This activity is all the more interesting since it appears to apply electively only to tetanus toxin. Diphtheria toxin for instance, to which a high dose of tripotassium glycyrrhizate has been added (50 or 60 mg per cc) does not undergo appreciable neutralization.

According to this observation it seemed of interest to study the product of hydrolysis of glycyrrhizin separately. It is known that when boiled with 3% sulfuric acid this molecule breaks down while producing two molecules of glucuronic acid $C^6H^{10}O^7$, very readily soluble in water, and one molecule of glycyrrhitic acid $C^{31}H^{45}O^3(OH)^2COOH$, which is insoluble. These tests have led to the following extremely clear result. On the one hand the neutralizing power of glucuronic acid is virtually zero(2).

(2) It would be more accurate to speak of uronic acid without definitely specifying glucuronic acid.

On the other hand all the neutralizing power of the primary glycyrrhizin molecule is found again quantitatively in glycyrrhitic acid, in other words 5 mg still neutralizes 800 lethal doses of tetanus toxin(3).

(3) Glycyrrhitic acid was solubilized with an excess of piperazine. I wish to express my thanks to Poirot who obtained the pure samples used in this work.

It must be concluded from this that these very definite cryptotoxic properties of soluble glycyrrhizates are not linked

to the presence of glucuronic acid in their molecule. The contrary might a priori have been supposed, either in view of the fact that this acid has an aldehyde function, or by analogy with the role it is said to play in vivo in certain mechanisms of antitoxic defense.

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Uncoupling of oxidative phosphorylation by glycyrrhetic acid, fusidic acid and some related triterpenoid acids

M. W. WHITEHOUSE*, P. D. G. DEAN† AND T. G. HALSALL

Some new derivatives of 18α- and 18β-glycyrrhetic acid and oleanolic acid were tested for their ability to inhibit phosphorylation coupled to succinate oxidation in rat liver mitochondria. Glycyrrhetic acid is a potent uncoupler of oxidative phosphorylation (approaching 2,4-dinitrophenol in potency): uncoupling activity is dependent upon each of the following functional groups: 3-hydroxy, 11-oxo and 30-carboxyl groups. Inversion of the configuration at C-18 (D/E ring junction) or replacement of the 11-oxo-12-ene system in ring C by the 9(11),12-diene system in glycyrrhetic acid abolished uncoupling activity. By contrast, the hemisuccinates (3-*O*-carboxypropionyl derivatives) of 18α-glycyrrhetic acid and of the 18β-9,12-diene acid were moderately potent uncoupling agents but less active than 18β-glycyrrhetic acid. The uncoupling activity of these hemisuccinates could not be attributed to the carboxypropionyl group alone. Structural analogues of various glycyrrhetic acid derivatives prepared from oleanolic acid (with the carboxyl group at C-28) were less active in uncoupling oxidative phosphorylation than the corresponding compounds in the glycyrrhetic acid series (with the carboxyl group at C-30). The uncoupling activity of some derivatives of two naturally occurring tetracyclic triterpenoid acids, polyporenic acid A and fusidic acid, was also investigated and found to largely depend upon their chemical structure. The possible application of these compounds as drugs in man is discussed.

GLYCYRRHETIC acid (glycyrrhetinic acid, Fig. 1A), the aglycone of glycyrrhizin (from liquorice) uncouples oxidative phosphorylation, that is, it inhibits the mitochondrial biosynthesis of adenosine 5′-triphosphate without inhibiting mitochondrial respiration.

Polyporenic acid A (ungulinic acid) is a tetracyclic triterpenoid (trimethyl sterol), one of several polyisoprenoids produced by the birch fungus *Polyporus betulinus* (Jones & Halsall, 1954; Fieser & Fieser, 1959; Ourisson, Crabbe & Rodig, 1964). It is 3α,12α-dihydroxy-24-methylene-5α,25ξ-lanost-8-en-26-oic acid (Fig. 1B) (Halsall, Hodges & Jones, 1953; Halsall & Hodges, 1954). Polyporenic acid A uncouples oxidative phosphorylation in liver mitochondria (Whitehouse, 1963) but does not exhibit anti-inflammatory activity in rats when assayed by the carrageenan granuloma test (private communication from Dr. R. M. A. Atkinson, Glaxo Laboratories).

Fusidic acid (Fusidin) is an antibiotic originally obtained from *Fusidium coccineum* (Godtfredsen, Roholt & Tybring, 1962; Newman, Bhat & others, 1962) and subsequently shown to be identical with ramycin, isolated from several cephalosporia and a phycomycete (Vanderhaeghe, Van Dijck & De Somer, 1965). Most features of its structure have now been elucidated (Fig. 1C) (Godtfredsen & Vangedal, 1962; Arigoni,

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Daehne & others, 1963; Bucourt & Legrand, 1964; Godtfredsen, Daehne & others, 1965). It has recently been found to alleviate arthritis in man (Dr. W. O. Godtfredsen, private communication).

We have now examined the relationship between chemical structure and uncoupling activity of several derivatives and analogues of glycyrrhetic acid, polyporenic acid A and fusidic acid.

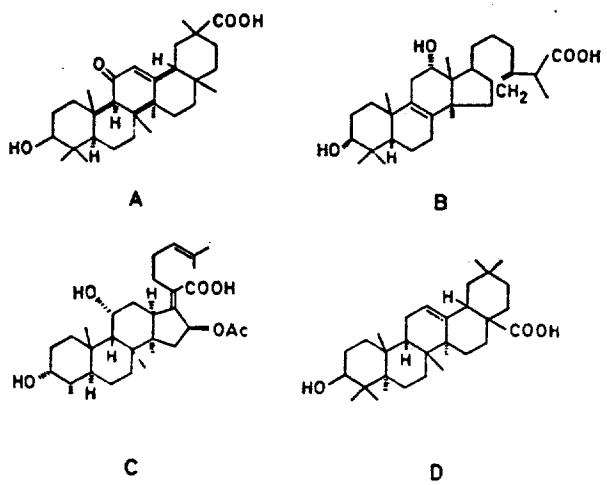

FIG. 1. Structure of some triterpenoid acids. A. 18β-Glycyrrhetic acid. B. Polyporenic acid A. C. Fusidic acid. D. Oleanolic acid.

Experimental

Materials. The following compounds were kindly made available by Dr. S. Gottfried & the late Prof. E. E. Turner, F.R.S. (Biorex Laboratories, London, E.C.1): oleanolic acid, purified 18α- and 18β-glycyrrhetic acid, the corresponding 3-*O*-hemisuccinate (β-carboxypropionyl) esters, the 3-*O*-sulphate ester (as the Na salt) of the 18β-acid, 3-dehydro- and 11-deoxo-18β-glycyrrhetic acid, 3-*O*-acetyl- and 3-*O*-propionyl-18β-glycyrrhetic acid, certain conjugates of oleanoic acid and glycyrrhetic acid with anthranilic acid and *p*-aminobenzoic acid, and a highly purified preparation of glycyrrhizin (originally obtained from Ihei Matsumoto & Co., Ltd., Tokyo). A commercial sample of ammonium glycyrrhizate was obtained from L. Light & Co., Colnbrook, Bucks.

Other derivatives of the glycyrrhetic acids and derivatives of oleanolic acid were synthesized as described (Dean, Halsall & Whitehouse, 1967).

Polyporenic acids and their derivatives were prepared according to Curtis, Heilbron & others (1953); Jones & Woods (1953) and Halsall & Hodges (1954). Eburicoic acid was isolated from *Polyporus eucalyptorura*. 3β-Hydroxycholest-5-en-26-oic acid was synthesized from 25-oxonorcholesterol (Dean & Whitehouse, 1966). Fusidic acid and some of its derivatives (Godtfredsen & Vangedal, 1962) were supplied by Dr. W. O. Godtfredsen (Leo Pharmaceutical Products, Copenhagen). Tumulosic acid was donated by Dr. J. E. E. Holker, University of Liverpool and cholesterol 3-*O*-(hydrogen sulphate) by Mr. P. R. H. Raggatt.

METHODS

Rat liver mitochondria were isolated and incubated at 30° with various oxidizable substrates, glucose and yeast hexokinase at pH 6·8 as detailed by Skidmore & Whitehouse (1965a). Oxygen uptake was measured manometrically; phosphate uptake was measured colorimetrically (Fiske & Subbarow, 1925). Compounds to be tested for their effect upon oxidative phosphorylation were added to the main compartment of Warburg vessels either in 100 μl of neutralized aqueous solution or in 25 μl of dimethylformamide together with the theoretical equivalent of aqueous sodium bicarbonate.

The activity of yeast hexokinase as a glucose phosphorylase in the presence of these triterpenoids was determined by the method of Darrow & Colowick (1955).

Drug binding to ϵ-amino-groups of bovine plasma albumen was determined colorimetrically using 2,4,6-trinitrobenzaldehyde (Skidmore & Whitehouse, 1965b; 1966); *N*-benzoyl-L-arginine ethyl ester was substrate.

Drug action on a thiol enzyme (papain) was studied by Whitehouse & Leader (1967).

Results

Glycyrrhetic acid (100 μM) and polyporenic acid A (200 μM) were fairly potent uncouplers of oxidative phosphorylation. A higher concentration of fusidic acid (600 μM) was needed for the same effect *in vitro*. At these concentrations these triterpenoid acids abolished the phosphorylation of ADP (i.e. uptake of inorganic phosphate) accompanying the mitochondrial oxidation of succinate, pyruvate, glutamate and citrate. Also, they had no effect on mitochondrial respiration or on either the glucose-phosphorylating or the ATP-ase activity (de la Fuente & Sols, 1963) of the yeast hexokinase which was added, with glucose, to the phosphorylating mitochondrial preparations to trap the newly incorporated labile γ-phosphate group of ATP (as the stable ester-phosphate group in glucose 6-phosphate). The uncoupling action of these acids was not reversed by co-incubation with 5 mM cysteamine or mercaptoethanol but was reversed on diluting the drug after preincubation with mitrochondria.

Further evidence that these acids were uncouplers of oxidative phosphorylation was found as follows. Slices of cattle tracheal cartilage were incubated with sodium [^{32}P]phosphate and with sodium [^{35}S]sulphate and the incorporation of ^{32}P into organic phosphates and the ATP-dependent incorporation of ^{35}S into the polysaccharide sulphates of the cartilage tissue were each determined in the presence and in the absence of these triterpenoids (Whitehouse & Haslam, 1962; Whitehouse, 1964a). Glycyrrhetic acid (100 μM) and polyporenic acid A (200 μM) each inhibited these biosynthetic processes by more than 50% without impairing the oxidation of [$^{14}C_u$]glucose and [$^{14}C_1$]octanoate to [^{14}C]carbon dioxide by these cartilage slices. Oleanolic acid (300 μM) and 11-deoxo-18β-glycyrrhetic acid (comp. 12; Table 1) (150 μM), which do not uncouple oxidative phosphorylation in liver mitochondria (see below), had no effect on either ^{32}P or ^{35}S incorporation by cartilage slices.

The relationship of chemical structure to uncoupling activity was explored with selected derivatives of glycyrrhetic, oleanolic, polyporenic and fusidic acids, using rat liver mitochondria incubated with succinate as the test system. A compound which lowered the P/O ratio to ⩽85% that of the P/O ratio obtained in parallel incubations without added triterpenoids, was considered to be capable of uncoupling oxidative phosphorylation. The P/O ratio in these drug-free controls (assigned a value of 100%) actually varied with each mitochondrial preparation but fell within the range 1·2–1·7 (see Skidmore & Whitehouse, 1965a).

With this biological test system, a fairly reproducible response was obtained to a given concentration of a triterpenoid. Thus in duplicate assays for 75 μM glycyrrhetic acid with 20 successive mitochondrial preparations, succinate oxidation was inhibited by no more than 20% and the P/O ratio was 32 $\pm$ 7 (s.e.m.)% of the controls; the range of P/O values being actually 10–55% of the controls.

UNCOUPLING ACTIVITY OF GLYCYRRHETIC ACID DERIVATIVES

Table 1 indicates the relative uncoupling potencies of 18β-glycyrrhetic acid and 17 of its derivatives. Simple derivatives lacking the ionized carboxyl group at C-30 were too water-insoluble for testing. Several

TABLE 1. UNCOUPLING ACTIVITY OF SOME DERIVATIVES OF 18α- AND 18β-GLYCYRRHETIC ACID AND RELATED COMPOUNDS

No.	Compound	Conc. $\cdot 10^{-4}$M	% Control P/O ratio
—	None	—	100
1	18α-Glycyrrhetic acid(isoglycyrrhetic acid)	1·5	92
2	18β-Glycyrrhetic acid	0·75	35
		1·5	5
3	3-Dehydro-18β-glycyrrhetic acid	0·75	83
4	Methyl 18β-glycyrrhetate	1·0*	86
5	3-*O*-Acetyl-18β-glycyrrhetic acid	0·75	73
		1·5	38
6	3-*O*-Propionyl-18β-glycyrrhetic acid	0·75	85
7	3-*O*-(β-Carboxypropionyl)-18α-glycyrrhetic acid	2·2	0
		1·2	43
8	3-*O*-(β-Carboxypropionyl)-18β-glycyrrhetic acid	2·2	34
		1·2	70
9	Methyl 3-*O*-(β-carboxypropionyl)-18α-glycyrrhetate	1·2	84
10	Methyl 3-*O*-(β-carboxypropionyl)-18β-glycyrrhetate	1·5	100
11	3-*O*-Acetyl-18β-glycyrrhetamide	0·75	100
12	11-Deoxo-18β-glycyrrhetic acid	1·5	96
13	3-*O*-(β-Carboxypropionyl)-11-deoxo-18β-glycyrrhetic acid	2·0	80
14	9,11-Dehydro-11-deoxo-18β-glycyrrhetic acid	0·75	100
15	3-*O*-(β-Carboxypropionyl)-9,11-dehydro-11-deoxy-18β-glycyrrhetic acid	2·0	5
		1·0	25
16	Glycyrrhizin (purified Na salt)	7·5	92
17	Glycyrrhizin (commercial NH_4^+ salt)	5·0	74
18	18β-Glycyrrhetic acid 3-*O*-(hydrogen sulphate)	2·5	0–70†
19	*N*-(18β-Glycyrrhetyl)glycine	1·5	65
20	*o*-(18β-Glycyrrhetamido)benzoic acid	1·5	53
21	*o*-[3-*O*-(β-Carboxypropionyl)-18β-glycyrrhetamido]benzoic acid	0·75	55
22	*o*-(3-*O*-Acetyl-18β-glycyrrhetamido)benzoic acid	0·75	30
		0·4	55
23	*p*-(3-*O*-Acetyl-18β-glycyrrhetamido)benzoic acid	0·75	95
24	*o*-Cholamidobenzoic acid	4·0	45
25	Cortisone 21-sulphate (Na salt)	2·5	98
26	Cholesterol 3-*O*-(hydrogen sulphate) (K salt)	2·5	100

* Conc. uncertain due to limited solubility.
† Variable activity, see text.

other derivatives, which retained this carboxyl group, were too insoluble in the presence of magnesium ions (4·5 mM) at pH 6·8 to be tested adequately. In either instance, when this happened with compounds containing a 3β-hydroxyl group, it was usually possible to test their 3-*O*-(β-carboxypropionyl derivatives ("hemisuccinate esters") for uncoupling activity instead. these particular derivatives being much more soluble than their parent alcohols in aqueous media containing magnesium ions.

3-*O*-(β-Carboxypropionyl)-18β-glycyrrhetic acid (comp. 8) was approximately one-third as potent as uncoupling agent as the parent alcohol, i.e. 18β-glycyrrhetic acid (comp. 2). The corresponding hemisuccinate of 18α-glycyrrhetic acid (comp. 7) was more active in uncoupling oxidative phosphorylation than the hemisuccinate of the acid (comp. 8). By contrast, the parent alcohol (comp. 1) was inactive; it was certainly sufficiently water-soluble in the presence of magnesium ions at pH 6·8 to be compared with the unsubstituted 18β-acid (comp. 2). In two other instances it was likewise found that hemisuccinates uncoupled oxidative phosphorylation (comp. 13 and 15), but the corresponding 3-hydroxy compounds did not (comp. 12 and 14).

Some "quasi-physiological" partition experiments were made in attempts to find an explanation for these findings. The removal of ultraviolet absorbing 3-hydroxy compounds and hemisuccinates from the aqueous salt solution pH 6·8 by liver mitochondria during a 30 min incubation at 30° was measured as follows. The residual ultraviolet absorption at 250 mμ [due to the 11-oxo-12-ene and the 9(11),12-diene chromophores respectively] in the aqueous phase was determined after removal of the mitochondrial lipid phase (at 8,500 *g*, 15 min) and subsequent partition (concentration) of the ultraviolet absorbing triterpenoids into methylene dichloride at pH 6·8; parallel assays were made on solutions of the triterpenoids similarly incubated without mitochondria.

These experiments showed that the parent 18α- and 18β-acids were both removed from the aqueous phase to approximately the same extent (ca. 80%) by thick mitochondrial suspensions (ca. 20% by volume); only 20% of the hemisuccinate of the 18α-acid was removed under the same conditions. Whilst these results indicate why the uncoupling activity of the hemisuccinate of the 18β- acid was only 1/4 to 1/3 that of the parent 18β-acid, they give no indication why the parent 18α-acid should be inactive while its hemisuccinate is a potent uncoupling agent. The hemisuccinate group itself apparently had no uncoupling activity; at least 5 of the other hemisuccinates of triterpenes tested had no effect upon oxidative phosphorylation (see below).

Replacement of the ionized carboxyl group at C-30 effectively abolished uncoupling activity; the methyl esters of 18β-glycyrrhetic acid (comp. 4) and of the hemisuccinates of 18α- and 18β-glycyrrhetic acids (comp. 9 and 10) and the amides of both the 18β-glycyrrhetic acid and the 3-*O*-acetyl-18β-acid (comp. 11), were each without any effect on oxidative phosphorylation [18β-glycyrrhetamide was not pure: it was tested in admixture with methyl glycyrrhetate (which is inactive) from which it was prepared by ammonolysis in liquid ammonia; the amide has a higher

Rf value than the ester on thin-layer plates developed with ethyl acetate].

Other more soluble derivatives of glycyrrhetinamide were also examined for uncoupling activity. Only the *N*-anthranilic acid (comp. 20) and *N*-glycine conjugates (comp. 19) had any significant activity. Esterification of the 3-hydroxyl group of the anthranilic acid conjugate (to give comp. 21 and 22) actually increased the uncoupling activity, perhaps by increasing the solubility.

Modification of the 3β-hydroxyl group of 18β-glycyrrhetic acid, either by oxidation to the ketone (comp. 3) or by esterification (comp. 5,6,8 and 18) diminished but did not abolish the uncoupling activity. The 3-*O*-acetyl, 3-*O*-(hydrogen sulphate) and 3-*O*-(β-carboxypropionyl) derivatives (comp. 5, 18, 8), though differing greatly in the ease with which they partition between liver mitochondria and aqueous solutions of pH 6·5 (the acetyl ester resembling the unesterified acid, see above) were all less active than the parent 3β-hydroxyl compound (comp. 2). The hydrogen sulphate showed variable uncoupling activity (relative to the parent acid: comp. 2) in different experiments, which may reflect some hydrolysis to the parent acid. This ester is labile, particularly in acidic media (Dr. J. C Turner, private communication). The very water-soluble 3-glycoside, glycyrrhizin (comp. 16 and 17) was inactive. These observations indicate that the presence of an unsubstituted hydroxyl function at C-3, though not essential for uncoupling activity, nevertheless determines the absolute activity of this type of compound in uncoupling oxidative phosphorylation.

Removal of the 11-keto-group of 18β-glycyrrhetic acid, with concomitant reduction of C-11 to a methylene group, virtually abolished the uncoupling activity (cf. comp. 12 and 13 with comp. 2 and 8). However, when the 11-oxo-12-ene grouping in 18β-glycyrrhetic acid was replaced by the 9(11),12-diene grouping the uncoupling activity was apparently retained in one instance (cf. comp. 15 with comp. 8) but not in another (cf. comp. 14 and 2). This situation corresponded to that found with the hemisuccinate of glycyrrhetic acid (active) and the unsubstituted 18α-acid (inactive); in each instance the parent 3β-hydroxy compounds were inactive whilst the 3-carboxypropionyl esters were active.

UNCOUPLING ACTIVITY OF OLEANOLIC ACID DERIVATIVES AND OTHER PENTACYCLIC ACIDS

Oleanolic acid (comp. 27, Table 2) is an isomer of 11-deoxo-18β-glycyrrhetic acid in which the carboxyl group is at C-28 (in the junction of the D and E rings) instead of at C-30 as in the glycyrrhetic acid series.

Neither 18β-oleanolic acid nor its hemisuccinate (comp. 28) uncoupled oxidative phosphorylation (Table 2). The hemisuccinate of 11-deoxo-glycyrrhetic acid (comp. 13), though non-absorbing at 250 mμ (the 11-oxo-12-ene grouping was absent) always showed some slight but significant uncoupling activity. Other analogues of active compounds in the glycyrrhetic acid series were prepared from oleanolic acid, e.g. 11-oxo-18β-oleanolic acid, for comparison with 18β-glycyrrhetic acid (Fig. 1A). These oleanolic acids (comp. 29, 30, 31, Table 2) were less potent uncoupling agents than the corresponding glycyrrhetic acids

TABLE 2. UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY SOME OLEANOLIC ACID DERIVATIVES

No.	Compound	Conc. $\times 10^{-4}$M	P/O ratio (% controls)
—	None	—	100
27	18β-Oleanolic acid	1·5	100
28	3-*O*-(β-Carboxypropionyl)-18β-oleanolic acid	3	92
29	11-Oxo-18β-oleanolic acid	1·5	83
30	3-*O*-Acetyl-11-oxo-18β-oleanolic acid	1·5	83
31	3-*O*-(β-Carboxypropionyl)-11-oxo-18β-oleanolic acid	2·2	72
32	3-*O*-(β-Carboxypropionyl)-9,11-dehydro-18β-oleanolic acid	2	15
33	3-*O*-(β-Carboxyproprionyl)11-oxo-oleanolyl (28 → 13)lactone	2	100
34	*o*-(Oleanolamido)benzoic acid	1·5	45

(comp. 1, 5, 8, 15, Table 1). These findings show that the position of the carboxyl group in this series of compounds (4 oleanolic acids and 4 glycyrrhetic acids) largely governs the uncoupling activity. The most active oleanolic acid analogue encountered in this study was the hemisuccinate of 9,11-dehydro-18β-oleanolic acid (comp. 32) which contains the 9(11),12-diene system.

The inactivity of the water-soluble lactone (comp. 33) again shows the requirement for an unsubstituted carboxyl group for uncoupling activity.

Other pentacyclic triterpenoid acids tested (ursolic, boswellic, morolic and 11-oxoboswellic acids) did not uncouple oxidative phosphorylation when tested at 150 μM.

UNCOUPLING ACTIVITY OF UNSATURATED STEROIDS AND TRITERPENOIDS

Some conjugated unsaturated ketones and water-soluble conjugated dienes were tested but found not to uncouple phosphorylation linked to succinate oxidation. Compounds tested included cholest-4-en-3-one (70 μM), 11-deoxycorticosterone (deoxycortone) (100 μM), cortisol-21-aldehyde (hydrocortisone 21-aldehyde) (200 μM), 3-β-carboxypropionyl)-ergosterol (100 μM). Uncoupling activity is evidently not associated with these unsaturated groups *per se*, in the absence of a nuclear carboxyl group. Other steroid sulphates (comp. 25, 26) did not mimic glycyrrhetic acid 3-*O*-sulphate (comp. 18) in uncoupling phosphorylation (Table 1).

UNCOUPLING ACTIVITY OF SOME TETRACYCLIC TRITERPENOID ACIDS

A few derivatives of polyporenic acid A, a βγ-unsaturated acid, were examined for uncoupling activity (Table 3). One of the αβ-unsaturated

TABLE 3. UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY SOME TETRACYCLIC TERPENOID (TRIMETHYL STEROID) ACIDS

No.	Compound	Conc. $\times 10^{-4}$M	P/O ratio (% control)
35	Polyporenic acid A	1·5	30
36	Polyporenic acid C	3	100
37	Eburicoic acid	3	100
38	Fusidic acid	3	75
39	ψ-Polyporenic acid (25-isopolyporenic acid)	5	45
40	3,12-Dehydropolyporenic acid	1·5	40
41	24,28-Dihydropolyporenic acid A	1·5	50
42	3β-Hydroxycholest-5-en-26-oic acid	1·5	55
43	Tumulosic acid	3	92

isomers ψ-polyporenic acid (comp. 39) and 24,28-dihydropolyporenic acid (comp. 41) were both less active than polyporenic acid A in uncoupling oxidative phosphorylation, indicating that the double bond between C-24 and C-28 may possibly contribute to the uncoupling activity of polyporenic acid A. The diketo-derivative (comp. 40) was less active than polyporenic acid A (cf. 3-dehydro-18β-glycyrrhetic acid and 18β-glycyrrhetic acid itself (comp. 3, 2)]. A crude preparation of the naturally occurring conjugate (glycoside?) of polyporenic acid A (containing a small quantity of the free acid) was much less potent than polyporenic acid A itself on a weight for weight basis. Unless this unknown congugate has a molecular weight at least 5 times that of the free acid, this observation suggests that the naturally occurring form(s) of polyporenic acid A is less active in uncoupling phosphorylation than the free acid (cf. glycyrrhizin and glycyrrhetic acid).

Polyporenic acid C (16α-hydroxy-24-methylene-3-oxo-5α-lanosta-7,9(11)-dien-20-oic acid) and tumulosic acid (3β,16α-dihydroxy-24-methylene-5α-lanost-8-en-20-oic acid), which are both also obtained from *Polyporus betulinus*, and eburicoic acid (3β-hydroxy-24-methylene-5α-lanost-8-en-20-oic acid) did not uncouple oxidative phosphorylation at concentrations at which they were soluble in the buffered salt medium.

Little variation in uncoupling activity was found when the fusidic acid derivatives described by Godtfredsen & Vangedal, (1962) were tested.

RELATIVE BINDING TO ALBUMEN

There was some correlation between the solubility of a terpenoid acid in the presence of 0·1 mM bovine plasma albumen and its uncoupling activity, for example eburicoic acid, oleanolic acid, 18α-glycyrrhetic acid and 11-deoxo-18β-glycyrrhetic acid did not bind sufficiently strongly to albumen to give 1 mM solutions but polyporenic acid A, 18β-glycyrrhetic acid and fusidic acid readily gave mM solutions in the presence of 0·1 mM.

TABLE 4. RELATIVE BINDING OF SOME ACIDIC POLYISOPRENOIDS TO ALBUMEN (LYSYL) AMINO-GROUPS DETERMINED COLORIMETRICALLY WITH 2,4,6-TRINITROBENZALDEHYDE. Inhibitors by 1 mM drug of colour development when reagent (0·1 mM) reacts with bovine plasma albumen (0·1 mM) at pH 7·4 (0·1M phosphate), determined at 425 mμ.

No.	Compound	Inhibition %
—	None	—
2	18β-Glycyrrhetic acid	35
3	3-Dehydro-18β-glycyrrhetic acid	40
5	3-*O*-Acetyl-18β-glycyrrhetic acid	38
19	*N*-(18β-Glycyrrhetyl)glycine	23
18	18β-Glycyrrhetic acid 3-*O*-(hydrogen sulphate)	48
16	Glycyrrhizin (purified Na salt)	0
17	Glycyrrhizin (commercial NH_4^+ salt)	0
8	3-*O*-(β-Carboxypropionyl)-18β-glycyrrhetic acid	60
21	*o*-[3-*O*-(β-Carboxypropionyl)-18β-glycyrrhetamido]benzoic acid	74
7	3-*O*-(β-Carboxypropionyl)-18α-glycyrrhetic acid	70
31	3-*O*-(β-Carboxypropionyl)-11-oxo-18β-oleanolic acid	75
35	Polyporenic acid A	65
41	24,28-Dihydropolyporenic acid A	44
38	Fusidic acid	20
	3,7,12-Trioxocholanic acid	0
	Deoxycholic acid	38
	3,12-Dioxocholanic acid	32

albumen. Within this latter class of albumen-soluble acids, uncoupling activity approximately paralleled the affinity of these compounds for the albumen (lysyl) ε-amino-groups as indicated by their ability to prevent 2,4,6-trinitrobenzaldehyde from reacting with these amino-groups and so quenching the red colouration formed by the albumen-trinitrobenzaldehyde interaction. [A similar correlation has been found in studies of aromatic anti-inflammatory acidic drugs (Skidmore & Whitehouse, 1965b).] Table 4 shows that this albumen-binding is primarily a property of the 30-carboxyl group in the glycyrrhetic acid series but is enhanced by the 3-(β-carboxypropionyl) (hemisuccinate) and 3-*O*-sulphate groups. The 3-*O*-(β-carboxypropionyl)-18α-glycyrrhetic acid (comp. 7) which is more potent than the hemisuccinate of the 18β-acid (comp. 8) in uncoupling phosphorylation, also binds more strongly to these albumen amino-groups. 3-*O*-(β-Carboxypropionyl)-11-oxo-18β-oleanolic acid (comp. 31) binds more strongly to albumen amino-groups than 3-*O*-(β-carboxypropionyl)-18β-oleanolic acid (comp. 28) but neither of these compounds significantly uncouples phosphorylation, showing that albumen-binding is of itself no absolute guide to potential uncoupling activity, though it may provide an indication of the relative potency within a given series of compounds with intrinsic uncoupling activity.

When these uncoupling acids were co-incubated with bovine plasma albumen and phosphorylating mitochondria, it was found that 75 μM 18β-glycyrrhetic acid no longer uncoupled phosphorylation in the presence of 1 mg/ml albumen. The hemisuccinates of 18α- and 18β-glycyrrhetic acids (comp. 7, 8) and glycyrrhetic acid 3-*O*-sulphate (comp. 18) (all at 25 μM) and sodium fusidate (comp. 38) (500 μM) still retained some uncoupling activity in the presence of 5 mg/ml albumen and polyporenic acid A (120 μM) retained some activity in the presence of 2·5 mg/ml albumen, but the potency of these acids was reduced (approx. 50–70%).

Discussion

These studies have been limited by the poor solubility of many of the compounds, but it is possible to draw the following conclusions.

(1) The ability of certain triterpenoid acids to uncouple oxidative phosphorylation must now be added to the list of known biological properties of terpenes (reviewed by Martin-Smith & Khatoon, 1963). The anti-inflammatory activity of glycyrrhetic acid in small animals and the inhibition of neoplastic cell growth by the triterpenoid fractions from *Polyporus betulinus* (Utzig & Samborski, 1957; Wandokanty & Utzig, 1958) may depend upon the uncoupling of oxidative phosphorylation in the subcutaneous or neoplastic tissue by glycyrrhetic and polyporenic acid A respectively. The antibacterial activity of fusidic acid (Godtfredsen & others 1962), like that of halophenols, salicyclic acid and many natural products (antibiotics) such as gramicidin and usnic acid, may also be a consequence of its uncoupling activity. The uncoupling activity of fusidic acid exceeds that of salicylic acid (Whitehouse, 1964a) whilst that of glycyrrhetic acid actually approaches the activity of 2,4-dinitrophenol

(almost complete uncoupling at 50 μM) and certainly exceeds the uncoupling activity of the most potent steroidal (cholanic) acid of animal origin, namely deoxycholic acid (Lee & Whitehouse, 1965).

(2) The uncoupling activity (and solubility at pH 7) of these particular triterpenoid acids is dependent upon their chemical structure. It should therefore be distinguished from many of the other biological activities of terpenes which are structurally non-specific (Martin-Smith & Khatoon, 1963). This dependence of the uncoupling activity upon chemical structure distinguishes these triterpenoid acids from most aliphatic fatty acids, and possibly also arylacetic acid (Whitehouse, 1964b), the uncoupling activity of which appears to depend only upon solubility in a lipid phase and the presence of a carboxyl group.

Each of the functional groups of glycyrrhetic acid (3-hydroxy,11-oxo and 30-carboxy) apparently contributes to its uncoupling activity, the carboxyl group and double unsaturation in ring C (either an 11-oxo-12-ene or a 9(11),12-diene system) being apparently essential for this purpose. The much weaker uncoupling activity of the olean-28-oic isomers (oleanolic acid derivatives) demonstrates that the activity of the glycyrrhetic acid derivatives is not a structurally non-specific activity of a highly lipophilic acid; if this were so, the oleanolic acid derivatives and glycyrrhetic acid derivatives should be approximately equipotent. The difference in activity of 18α- and 18β-glycyrrhetic acid further emphasizes this.

Similar conclusions may be drawn from our rather more limited survey of the tetracyclic terpenoid acids. The presence of the double bond in polyporenic acid A and the 20-iso-configuration in fusidic acid may be of particular importance in determining uncoupling potency. It is remarkable that the ketones, derived by oxidation of the hydroxy-group in position 3 in glycyrrhetic acid and in position 12 in polyporenic acid A, should be so much less active than the parent alcohols. This parallels the observation that 3-, 12- (and 7-) oxocholanic acids are very much weaker uncoupling agents than the corresponding hydroxycholanic acids (Lee & Whitehouse, 1965). Oxidation of these hydroxy-acids to the corresponding ketones does not seem to alter the affinity for albumen amino-groups however.

The prospect of using triterpenoid acids as effective drugs in man (antibacterials or antirheumatic agents) is tempered by the fact that they may be strongly bound to plasma proteins *in vivo* and effectively rendered unavailable, just as we found to be the case with glycyrrhetic acid in the presence of serum albumen *in vitro*. Though this protein binding of the potential drug may be much diminished by increasing the water solubility of the triterpenoid, e.g. by β-carboxypropionylation, such modification of the molecule also diminishes or abolishes the uncoupling activity. Thus the highly water-soluble natural glycoside, glycyrrhizin, is virtually inactive. The relatively low uncoupling activity of the rather water-soluble fusidic acid (salts) and of 3-*O*-(β-carboxypropionyl)-18β-glycyrrhetic acid compares unfavourably with that of the less oxygenated and much less water-soluble polyporenic acid A or glycyrrhetic acids. This increase in water solubility must effectively lower the concentration

of the acid in, or across, lipid-rich phases such as the cell membranes and within the mitochondria themselves. Furthermore in view of the rapidity with which circulating cholanic acids and many steroid hormones are taken up and conjugated by the liver, to be subsequently excreted with the bile flow, it is likely that these higher polycyclic acids will suffer a similar fate. Parke, Pollack & Williams (1963) have already shown that 95% of a single dose of radioactive glycyrrhetic acid administered intraperitoneally to rats is excreted via the bile within 6 to 8 hr, mainly as three metabolites.

One of these has now been shown to be the 3-*O*-(hydrogen sulphate) ester of glycyrrhetic acid (Iveson, Parke & Williams, 1966). This particular metabolite (comp. 18) does uncouple oxidative phosphorylation, even in the presence of plasma albumen, although it is less potent than glycyrrhetic acid itself in the absence of albumen. The glycine congugate of glycyrrhetic acid (comp. 19) was synthesized and found to be much less potent in uncoupling phosphorylation than glycyrrhetic acid itself. This conjugate does not seem to be formed in the rat; it is actually less polar than any of the glycyrrhetic acid metabolites detected by Parke & others (1963) in rat bile (Dr. D. V. Parke, private communication).

The only compounds that were more potent uncoupling agents than glycyrrhetic acid and, therefore, of potential interest as novel anti-inflammatory drugs, were the conjugates with anthranilic acid (comp. 20, 21, 22). That this was largely due to the anthranyl pharmacophore rather than the glycyrrhetyl moiety was indicated by (*a*) the feeble uncoupling activity of the analogue conjugated with *p*-aminobenzoic acid (comp. 23) and (*b*) the uncoupling activity of other *N*-acylanthranilic acids (Leader & Whitehouse, 1966; Whitehouse, 1964a and unpublished observations), including *o*-(oleanolamido)benzoic acid [*N*-(oleanoyl)anthranilic acid, comp. 34].

All these terpenoid acids appear to uncouple phosphorylation by interaction with key lysyl amino-groups participating in mitochondrial phosphorylation. There was a correlation between their ability to bind to these same groups on plasma albumen and their uncoupling potency *in vitro*. Neither the hemisuccinates of 18α- or 18β-glycyrrhetic acid (comp. 7, 8) nor fusidic acid inhibited papain (a thiol enzyme), when tested at 1 mM in the presence of 0·25 mM mercaptoethanol; the uncoupling activity of these acids and polyporenic acid A was not reversed by thiols, so it is unlikely that they uncouple phosphorylation merely by interaction with essential thiol groups in mitochondria. [This is an alternative mechanism of uncoupling phosphorylation and explains the uncoupling activity of ninhydrin (Whitehouse & Leader, 1967)].

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