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CONTENTS

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Effect of Adrenalectomy on the Course of Alloxan Induced Diabetes in Albino Rats

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Studies on the effect of adrenalectomy in albino rats, before and after administration of alloxan, have shown that fasting blood sugar in bilateral adrenalectomized rats remains at a lower level than in normal rats. Glucose tolerance tests have shown increased tolerance of adrenalectomized rats to glucose as compared to normal rats. Pancreas of adrenalectomized rats, 12 days after adrenalectomy, shows the formation of some small islands of Langerhans. The beta cells of the small islands appear darker (suggestive of more granules and hence more insulin content) than the beta cells of the bigger island in the same section. Bilateral adrenalectomy, prior to administration of alloxan, protects the rats from the diabetogenic action of alloxan. Bilateral adrenalectomy in alloxan diabetic rats also causes amelioration of their diabetic condition. Glucose tolerance tests, carried out with animals recovering from diabetic condition, have shown that tolerance to glucose in animals adrenalectomized before alloxan administration is better than the tolerance of animals adrenalectomized after alloxan administration.

THE exact mechanism which initiates the diabetic state in man is not definitely known. Factor or factors involved in this mechanism may be multiple and variable. But in diabetes there exists a defect in the utilization of carbohydrate and this defect is due to the lack of normal activity of insulin. The insufficient activity of insulin may be due to various reasons such as diminished production of insulin, increased destruction of insulin or inactivation of insulin by some antagonistic substances. In some instances it seems that insulin insufficiency is relative and not absolute and there actually may be normal amount of insulin produced but its effect is neutralized to a greater or lesser degree by some antagonists[1]. In many instances diabetic syndrome is secondary to disturbances in endocrine glands other than pancreas. Of these, the important ones are the anterior lobe of pituitary and the adrenal cortex which may be related to the production of diabetes mellitus as was strongly suggested by the classical work[2,3].

Removal of adrenals has been reported to reduce blood sugar[4,5]. Amelioration of diabetic state following adrenalectomy was confirmed[3,6]. Lukens and Dohan[7] and Long *et al.*[8] have observed that glycosuria did not occur in pancreatectomized and adrenalectomized rats but glycosuria appears if corticosteroids are given to such animals. The present investigation was undertaken to study the diabetogenic action of alloxan in previously bilaterally adrenalectomized albino rats. Effect of bilateral adrenalectomy in alloxan diabetic rats has also been recorded for comparison.

Materials and methods

Both male and female adult albino rats of the C.D.R.I. colony were used in all the experiments. The weight of the rats varied from 140 to 170 g.

Male and female rats were housed in separate cages. The animals were kept under observation on the same diet (bread, gram, green fruits, root vegetables, fresh milk and water *ad lib.*) for a period of 10-15 days. Pancreas from 3 rats was collected for histological study (normal). The animals were then divided into two groups. Group I animals consisted of 100 rats. Glucose tolerance test was carried out on these animals. Bilateral adrenalectomy was then performed in 70 of these animals under ether anaesthesia at one stage using dorsal approach. Adrenalectomized animals were maintained by subcutaneous injection of 1 per cent NaCl for the first few days after adrenalectomy and thereafter by providing the same saline solution as drink in addition to regular water supply. The remaining 30 normal (control) animals were sham adrenalectomized. Twelve to 15 days after adrenalectomy, pancreas from 2 of the 57 animals which continued to survive and of 7 (pancreas taken out immediately after death) of the 13 animals which died within 15 days of adrenalectomy) were collected for histological study. The rest 55 adrenalectomized and 30 sham-operated animals were subjected to a second glucose tolerance test. After 15 days of adrenalectomy alloxan monohydrate in the dose of 4-5 mg./100 g. body weight in 10 per cent solution was administered through the tail vein to all the 55 adrenalectomized animals. The 30 sham-operated animals were injected with the same amount of solvent (distilled water). On the same day another group (group II) of 50 normal animals were injected with alloxan monohydrate, following the same procedure as in adrenalectomized animals. Eight of the animals died within 7 days of alloxan administration. Of the surviving 42 animals, bilateral adrenalectomy on 27 and sham operation on 15 animals were performed after 15 days of alloxan administration. Five animals died after adrenalectomy. Only 37 alloxanized animals (22 adrenalectomized and 15 sham operated) survived for further observation.

Blood sugar of the animals was determined before and then at any time necessary within 2 hr after alloxan administration. Insulin treatment to alloxanized diabetic animals was discontinued from the day of adrenalectomy.

Urine of all the alloxanized animals (both adrenalectomized and adrenal intact) was examined daily for sugar and their blood sugar was determined once a week. Animals showing persistent glycosuria (+++) and hyperglycaemia above 200 mg. per cent at the end of 2 weeks after alloxan administration were considered in a state of permanent diabetes. All the animals during the whole experimental period were maintained on the same diet and 1 per cent NaCl and water for drink.

Animals were kept without food (water and saline *ad lib.*) for 18-20 hr under the following circumstances: (i) before glucose tolerance test, (ii) before adrenalectomy, (iii) before alloxan administration, and (iv) before single blood sugar determination. Insulin administration to animals receiving insulin treatment was stopped 24 hr before blood sugar determination. Glucose tolerance test was done according to the method of Cole and Harned[9]. Blood sugar was determined by Nelson's micromethod[10]. At the conclusion of the experiment, pancreas of the following categories of animals was collected for histological study: (i) adrenalectomized alloxanized animals, (ii) alloxanized adrenalectomized animals, and (iii) alloxan diabetic animals. Sections for histological study were prepared according to Gomori's aldehyde fuchsine method[11].

Results

Glucose tolerance before and after adrenalectomy (Fig. 1 and Tables 1 and 2) — Fasting (18-20 hr) blood sugar ($96{\cdot}2 \pm 3{\cdot}1$ mg. per cent) in adrenalectomized animals was lower than the blood sugar ($126{\cdot}6 \pm 1{\cdot}03$ mg. per cent) in animals with intact adrenals. After administration of glucose, the maximum rise of blood sugar in adrenalectomized animals ($218{\cdot}1 \pm 5{\cdot}8$ mg. per cent) reached a higher level than the blood sugar in adrenal intact condition ($194{\cdot}3 \pm 4{\cdot}2$ mg. per cent). The return of blood sugar thereafter towards the preglucose level ($100{\cdot}7 \pm 1{\cdot}5$ mg. per cent) was quicker and more or less complete at the end of 2 hr after glucose administration in adrenalectomized animals. In adrenal intact condition, however, the return of blood sugar was delayed and at the end of 2 hr

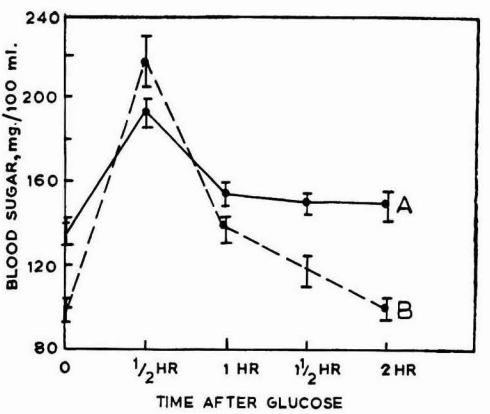

Fig. 1 — Glucose tolerance test on normal (A) and adrenalectomized (B) rats

TABLE 1 — GLUCOSE TOLERANCE TEST WITH NORMAL AND ADRENALECTOMIZED RATS (FIG. 1)

	Blood sugar, *mg./ml.*				Glycosuria after glucose administration
	Before glucose administration	After glucose administration			
		30 min.	60 min.	90 min.	120 min.	
Normal rats (av. of 100 rats)	126·6±1·03	194·3±4·2	158·2±3·6	150·1±3·0	150·5±2·3	nil
Adrenalectomized rats (av. of 55 rats)	96·2±3·10	218·1±5·8	139·1±3·5	119·6±1·6	100·7±1·5	Sugar (+ to ++) appeared in the urine of 50 rats within 60 min. after glucose administration but at the end of 2 hr, urine of all animals remained sugar free

TABLE 2 — GLUCOSE TOLERANCE TEST WITH RATS ADRENALECTOMIZED BEFORE AND AFTER ADMINISTRATION OF ALLOXAN AND SHOWING IMPROVEMENT IN DIABETIC CONDITION (FIG. 4)

	Blood sugar, *mg./ml.*				Glycosuria after glucose administration
	Before glucose administration	After glucose administration			
		30 min.	60 min.	90 min.	120 min.	
Batch A: Alloxan administered on 14th day after adrenalectomy (av. of 27 rats)	100·7±2·5	310·3±10·6	240·5±10·4	180·6±5·8	116·4±6·08	Present in urine collected during 30-90 min., but absent in urine after 2 hr
Batch B: Adrenalectomized on 15th day after alloxan (av. of 22 rats)	90·6±5·7	291·3±11·7	247·5±10·7	208·3±6·4	164·2±5·70	Present in urine collected during 30-90 min., but after 2 hr sugar was present in 15 rats and absent in 7

after glucose administration, it still remained higher (150·5 ± 2·3 mg. per cent) than preglucose level. Glycosuria appears in 50 out of 55 adrenalectomized animals within 30 min. after glucose administration but disappeared by the end of 2 hr. The results of glucose tolerance test in sham-operated animals were similar to those of normal (before operation) animals and are, therefore, not shown.

Effect of alloxan administration in normal and adrenalectomized animals — Marked glycosuria and hyperglycaemia were present in all the 42 normal animals within 15 days after alloxan. These animals showed rapid loss of weight during 10-12 days after alloxan administration.

Hypoglycaemic symptoms appeared in 52 out of 55 adrenalectomized animals within 15 min. to 1 hr after alloxan administration (Fig. 2). Twenty-five animals died due to sudden and severe hypoglycaemic shock. Three animals did not show any hypoglycaemic symptoms after alloxan administration and no glucose was given to them.

Glycosuria (++ to +++) and hyperglycaemia (av. 220 ± 6·1 mg. per cent) in all the 30 surviving animals appeared within 3-4 days after alloxan administration. Glycosuria disappeared in 27 animals and their blood sugar by the end of 4 weeks after alloxan, came down almost to the pre-alloxan

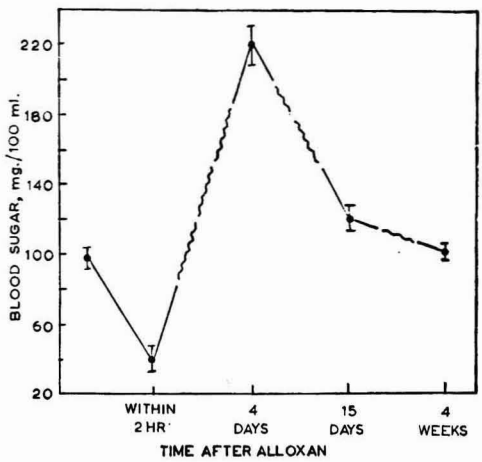

Fig. 2 — Variation of blood sugar in adrenalectomized rats after alloxan administration

level (av. 100·2 ± 3·1 mg. per cent). The 27 animals which recovered from diabetic condition showed increase in their body weight. Adrenalectomized animals showed greater growth than normal[12].

At the conclusion of the experiment 3 adrenalectomized mild diabetic rats and some of the

adrenalectomized rats which recovered from diabetic condition were dissected to examine whether any regeneration of removed adrenals had occurred. In the 3 animals a definite adrenal-like growth was observed. The growth appeared reddish in colour and round in shape. In 27 animals nothing was found to create any doubt against complete absence of adrenals.

Bilateral adrenalectomy and sham operation on alloxan diabetic animals (Fig. 3) — Glycosuria in the 22 alloxanized adrenalectomized animals disappeared completely and their blood sugar came down to normal within 3-4 weeks after adrenalectomy. Diabetic condition in 15 sham-operated animals remained more or less the same as before operation. Animals which recovered from diabetic condition after adrenalectomy began to gain in weight.

Glucose tolerance of adrenalectomized rats which showed improvement in diabetic condition (Fig. 4) — Twenty-seven bilateral adrenalectomized rats (batch A) recovered from diabetic condition produced by the administration of alloxan. Twenty-two alloxan diabetic rats (batch B) showed amelioration in their diabetic condition after bilateral adrenalectomy. Fasting blood sugar in batches A and B of animals (100·7 $\pm$ 2·5 and 90·6 $\pm$ 5·7 mg. per cent respectively) was lower than normal and the maximum rise of blood sugar (310·3 $\pm$ 10·6 and 291·3 $\pm$ 11·7 mg. per cent) after glucose reached a much higher level than that observed in normal. The fall of blood sugar thereafter was comparatively greater in batch A animals. Two hours after glucose administration, blood sugar in batch A animals (116·4 $\pm$ 6·08 mg. per cent) reached almost the preglucose level, whereas in batch B animals, blood sugar (164·2 $\pm$ 5·7 mg. per cent) at the end of 2 hr after glucose administration still remained appreciably above the preglucose level. Glycosuria (+ to ++) within 30-90 min. after glucose administration appeared in all the animals but disappeared by the end of 2 hr except in 15 batch B animals which still showed the presence of sugar (traces to +).

Discussion

Diminished initial fasting blood sugar and higher peak values after administration of glucose in adrenalectomized animals is possibly due to reduced ability of adrenalectomized animals to maintain and store liver glycogen[4,5,8,13]. The quantity of insulin secreted by the pancreatic beta cells due to stimulation of elevated blood sugar[14] might be the same in both normal (adrenal intact) and adrenalectomized animals. In normal animals, at a

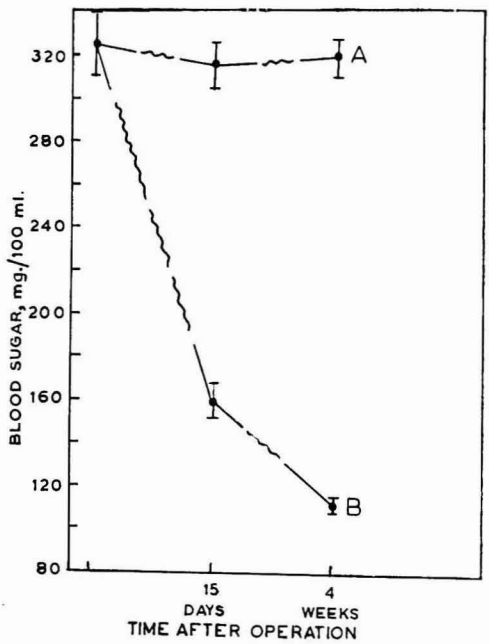

Fig. 3 — Variation of blood sugar in alloxan diabetic rats after sham operation and adrenalectomy [A: Sham-operated rats. B: Adrenalectomized rats]

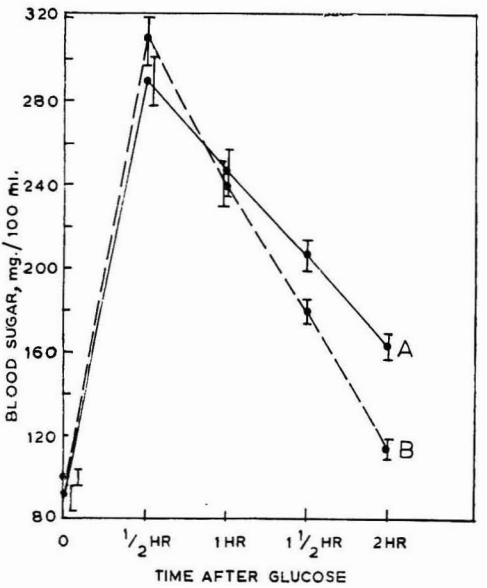

Fig. 4 — Glucose tolerance test on rats subjected to bilateral adrenalectomy and which showed improvement in their diabetic condition [A: Rats subjected to adrenalectomy prior to alloxan administration. B: Alloxan diabetic rats after adrenalectomy]

certain level of blood sugar a counteracting mechanism comes into play which checks the activity of insulin to prevent further rapid fall of blood sugar and thus maintains the blood sugar at the physiological level. Adrenal cortical hormones possibly play a role in this mechanism[15]. In adrenalectomized animals activity of insulin, liberated by the pancreatic beta cells, is possibly greater and lasts longer due to the absence of anti-insulin factor of adrenal cortical hormone and thus causes quicker and greater fall of blood sugar. Removal of adrenals has been observed to reduce the blood sugar and increase the tolerance to glucose[4,5,16].

Histological study of the pancreas obtained from normal and adrenalectomized animals 12 to 15 days after adrenalectomy shows that some small islands consisting of a few beta cells have appeared in the pancreas of adrenalectomized animals but no such island has been observed in the pancreas of normal (adrenal intact) animals (Fig. 5). Further, it has been observed that the beta cells of the small islands in the pancreas of adrenalectomized animals stained darker than the beta cells of larger islands in the same section of the pancreas. The darker appearance of the beta cells of the small islands possibly indicates the presence of more granules. The granules have been correlated to represent stored insulin[17]. So it is possible that in adrenalectomized animals some more insulin is available which may be an additional cause of greater fall of blood sugar. Adrenalectomy has been found to favour the histological and functional regeneration of beta cells of islands of Langerhans[18].

Hypoglycaemic phase, after alloxan administration, has been attributed to the liberation of preformed insulin from the damaged islands[19]. Severity of hypoglycaemic reaction after alloxan in adrenalectomized rats may be due to the following factors: (i) Insulin liberated from the beta cells of islands of Langerhans may remain active for longer period due to the absence of one of the anti-insulin factors, that is, adrenal cortical hormone; (ii) appearance of some new beta cells in the pancreas of adrenalectomized animals (Fig. 5A) is suggestive of the sources for the liberation of an increased amount of insulin; and (iii) diminished liver glycogen due to adrenalectomy may be a contributory factor[20]. The difference in time for returning the blood sugar towards the preglucose level in the two batches of animals is possibly due to the difference in quantity of insulin available in the two batches of animals. As the conditions, other than adrenalectomy and alloxan administration, were the same in both batches of animals, it is unlikely that the differences observed are due to some other reason

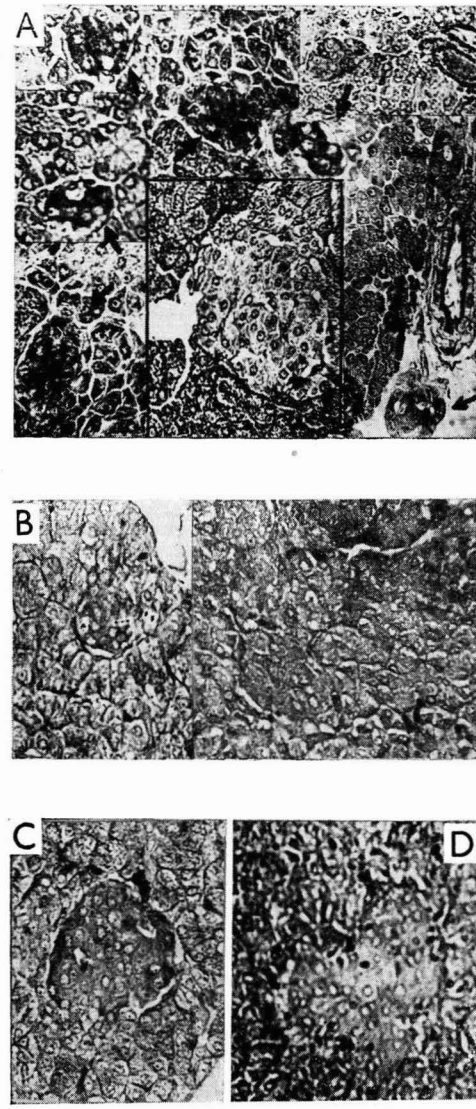

Fig. 5 — Photomicrographs (× 140) of pancreas of normal and adrenalectomized rats [A: The arrows point to the small islets (newly formed) in the pancreas of rats 12 days after adrenalectomy. The small islets appear darker than the larger island (centre). The dark appearance indicates the presence of more beta cell granules. B: Two small islands in the pancreas of adrenalectomized rat after recovery from alloxan diabetes. The beta cells appeared to be normal with cell outline and granules. C: Island in the pancreas of alloxan diabetic rat, after amelioration of diabetic condition following adrenalectomy; beta cells are fewer, but some central cells appeared to be intact with cell outline and granules. D: The island in the pancreas of diabetic rat. The island is irregular in shape. There is extensive necrosis of the beta cells with no cell outline; only some nuclei are visible. The sections were stained with Gomori's aldehyde fuchsine stain; photomicrographs in the case of A and B were taken from the same section]

or reasons. The only permanent effect of alloxan at the dose level used occurs on beta cells of the pancreas[21]. So it is possible that in batch A animals some of the newly formed beta cells (as has been observed to occur in adrenalectomized animals) may have escaped from the damaging action of alloxan (Fig. 5, B, C and D) and thus make more insulin available which perhaps explains the different behaviour of blood sugar in batch A animals. These findings support the view that adrenal cortex plays some important role in carbohydrate metabolism. Since the known function of ACTH is to increase the rate of acceleration of adrenal cortical hormone, imbalance between hypophysis and adrenal system may have some relation to the development of insular diabetes.

Summary

1. Initial fasting blood sugar in bilateral adrenalectomized rats is lower than that of normal rats.

2. The peak values of blood sugar, after glucose administration in adrenalectomized rats, though reached at the same time, are higher than those in normal rats. But the removal of sugar from circulation in adrenalectomized rats is quicker and greater than in normal rats.

3. Histological pictures of pancreas, 12-15 days after adrenalectomy, show the presence of many small islets consisting of a few beta cells. The beta cells of the small islets appeared darker than the beta cells of the bigger islands. The small islets are suspected to be new islets formed as a result of adrenalectomy.

4. Majority (about 90 per cent) of the adrenalectomized animals after alloxan administration recovered from diabetic condition and continued to be normal during the period of observation.

5. The results of glucose tolerance test carried out with animals, adrenalectomized before alloxan administration and subsequently recovered from diabetic condition, and with animals which became diabetic after alloxan administration but amelioration of their diabetic condition occurred after subsequent adrenalectomy, indicate increased sugar tolerance in animals adrenalectomized before alloxan administration.

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Studies in Hypoglycaemic Sulphonylureas

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Benzenesulphonylureas, having different substituents in the benzene nucleus as well as in the terminal nitrogen, have been synthesized from the corresponding thioureas by treatment with hydrogen peroxide. The compounds have been screened for hypoglycaemic activity in normal fasting rabbits, and fifteen of the compounds have shown good hypoglycaemic activity equivalent to or better than that of tolbutamide. Three compounds, viz. N-benzenesulphonyl-N′-isopropylurea, N-benzenesulphonyl-N′-*n*-butylurea and N-*p*-methoxybenzenesulphonyl-N′-cyclohexylurea, have shown pronounced and prolonged hypoglycaemic action; this finding has been confirmed by studies carried out at lower dose levels in rats and guinea-pigs.

EXTENSIVE clinical reports which have appeared during the past few years on three of the well-known sulphonylureas, viz. carbutamide, tolbutamide and chloropropamide, have revealed their shortcomings and indicated the need for further search to discover better compounds having lower toxicity and longer duration of action. Systematic study of hypoglycaemic activity of various N-N′-disubstituted sulphonylureas is not reported in literature. In many cases the activity is either not reported or where reported there has not been agreement on the results among the different workers. Moreover, the dose level studied by earlier workers was very high (about 100-300 mg./kg.[1]). This necessitated systematic investigation of these compounds. In this paper is reported the synthesis and the hypoglycaemic activity of new sulphonylurea derivatives. A few compounds whose synthesis have been reported but the activity data were not available have also been included in our studies.

We have found that with small increase in dosage, the hypoglycaemic activity of sulphonylureas increases but above a certain level further increase in dosage does not produce a rise in hypoglycaemic action. In some cases there is actually a reversal of the action with production of hyperglycaemia instead of hypoglycaemia. Higher dose level leading to hyperglycaemia has also been noted by Adami *et al.*[2] and Bander[3]. Because of these findings we have studied the action of the entire series at a lower dose level of 50 mg./kg. and the most promising compounds of this series retested at 25 mg. and 10 mg./kg.

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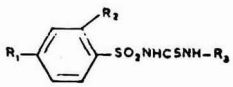
Experimental procedure

Synthesis of sulphonylureas — The sulphonylureas reported in this communication have been prepared from the corresponding sulphonylthioureas by treatment with hydrogen peroxide as described by Shah *et al.*[4]. The requisite sulphonylthioureas were prepared by condensing the appropriate arylsulphonamides with isothiocyanates in the presence of anhydrous potassium carbonate. By the use of anhydrous potassium carbonate in dry acetone, the thioureas were more conveniently isolated. In case of *p*-alkoxybenzenesulphonamides, chloroform has been used as solvent during chlorosulphonation and amides were prepared by treatment with ammonia as described by Huntress and Carten[5]. All the alkyl isothiocyanates were prepared from the corresponding alkylamines by the method of Dains *et al.*[6].

The details of the method followed for the synthesis of arylsulphonylthioureas and the corresponding ureas are illustrated by the following examples.

N-(4-*Methoxybenzenesulphonyl*)-*N′*-*cyclohexylthiourea* — A mixture of *p*-methoxybenzenesulphonamide (5·0 g.), anhydrous potassium carbonate (5·0 g.) and cyclohexylisothiocyanate (4·5 ml.) in acetone (100 ml.) was refluxed on a steam bath for about 10 hr. Acetone was then distilled off completely and the solid residue dissolved in water

TABLE 1 — N-N'-DISUBSTITUTED SULPHONYLTHIOUREAS



Sl No.	R_1	R_2	R_3	M.P. °C.	Mol. formula	Nitrogen, % Found	Nitrogen, % Reqd
1	H	H	Ethyl	120	$C_9H_{12}N_2O_3S_2$	12·02	12·17
2	H	H	*n*-Propyl	113-15	$C_{10}H_{14}N_2O_3S_2$	10·60	10·85
3	H	H	Isopropyl	102-3	$C_{10}H_{14}N_2O_3S_2$	10·65	10·85
4	H	H	Isobutyl	102	$C_{11}H_{16}N_2O_3S_2$	10·40	10·29
5	CH_3	H	do	94-95	$C_{12}H_{18}N_2O_3S_2$	9·55	9·79
6	H	CH_3	Methyl	125-6	$C_9H_{12}N_2O_3S_2$	11·56	11·46
7	H	CH_3	Ethyl	97-98	$C_{10}H_{14}N_2O_3S_2$	11·20	10·85
8	H	CH_3	*n*-Propyl	98-99	$C_{11}H_{16}N_2O_3S_2$	10·47	10·29
9	H	CH_3	Isopropyl	122	$C_{11}H_{16}N_2O_3S_2$	10·64	10·29
10	H	CH_3	Isobutyl	89-90	$C_{12}H_{18}N_2O_3S_2$	10·02	9·79
11	H	CH_3	Cyclohexyl	146	$C_{14}H_{20}N_2O_3S_2$	8·83	8·97
12	Cl	H	Methyl	160	$C_8H_9ClN_2O_3S_2$	10·98	10·58
13	Cl	H	Ethyl	146-8	$C_9H_{11}ClN_2O_3S_2$	10·05	10·05
14	Cl	H	Allyl	117-18	$C_{10}H_{11}ClN_2O_3S_2$	10·03	9·63
15	Cl	H	*n*-Propyl	135-6	$C_{10}H_{13}ClN_2O_3S_2$	9·38	9·58
16	Cl	H	Isopropyl	110-12	$C_{10}H_{13}ClN_2O_3S_2$	9·60	9·58
17	Cl	H	Isobutyl	112-13	$C_{11}H_{15}ClN_2O_3S_2$	9·01	9·13
18	CH_3O	H	Methyl	152-4	$C_9H_{12}N_2O_4S_2$	10·86	10·76
19	CH_3O	H	Ethyl	132-3	$C_{10}H_{14}N_2O_4S_2$	10·40	10·21
20	CH_3O	H	*n*-Propyl	104	$C_{11}H_{16}N_2O_4S_2$	9·62	9·72
21	CH_3O	H	Isopropyl	106-8	$C_{11}H_{16}N_2O_4S_2$	10·06	9·72
22	CH_3O	H	Isobutyl	96	$C_{12}H_{18}N_2O_4S_2$	9·52	9·27
23	CH_3O	H	Cyclohexyl	144-5	$C_{14}H_{20}N_2O_4S_2$	8·53	8·53
24	C_2H_5O	H	Methyl	159	$C_{10}H_{14}N_2O_4S_2$	9·83	10·21
25	C_2H_5O	H	Ethyl	134-6	$C_{11}H_{16}N_2O_4S_2$	9·62	9·72
26	C_2H_5O	H	*n*-Propyl	123-5	$C_{12}H_{18}N_2O_4S_2$	9·45	9·27
27	C_2H_5O	H	Isopropyl	133-4	$C_{12}H_{18}N_2O_4S_2$	9·16	9·27
28	C_2H_5O	H	Isobutyl	118-19	$C_{13}H_{20}N_2O_4S_2$	8·63	8·86
29	C_2H_5O	H	Cyclohexyl	158-60	$C_{15}H_{22}N_2O_4S_2$	8·28	8·18
30	C_3H_7O	H	Methyl	144-5	$C_{11}H_{16}N_2O_4S_2$	9·63	9·72
31	C_3H_7O	H	Ethyl	115-17	$C_{12}H_{18}N_2O_4S_2$	8·95	9·27
32	C_3H_7O	H	Allyl	101-2	$C_{13}H_{18}N_2O_4S_2$	8·77	8·91
33	C_3H_7O	H	*n*-Propyl	113-14	$C_{13}H_{20}N_2O_4S_2$	8·79	8·86
34	C_3H_7O	H	Isopropyl	109-10	$C_{13}H_{20}N_2O_4S_2$	8·69	8·86
35	C_3H_7O	H	*n*-Butyl	111-13	$C_{14}H_{22}N_2O_4S_2$	8·28	8·48
36	C_3H_7O	H	Isobutyl	108	$C_{14}H_{22}N_2O_4S_2$	8·40	8·48
37	C_3H_7O	H	Cyclohexyl	155-7	$C_{16}H_{24}N_2O_4S_2$	7·46	7·86
38	C_4H_9O	H	Methyl	128-9	$C_{12}H_{18}N_2O_4S_2$	8·97	9·27
39	C_4H_9O	H	Ethyl	101-2	$C_{13}H_{20}N_2O_4S_2$	8·66	8·86
40	C_4H_9O	H	Allyl	92-93	$C_{14}H_{20}N_2O_4S_2$	8·32	8·53
41	C_4H_9O	H	*n*-Propyl	110	$C_{14}H_{22}N_2O_4S_2$	8·56	8·48
42	C_4H_9O	H	Isopropyl	112-13	$C_{14}H_{22}N_2O_4S_2$	8·16	8·48
43	C_4H_9O	H	*n*-Butyl	103-4	$C_{15}H_{24}N_2O_4S_2$	8·01	8·14
44	C_4H_9O	H	Isobutyl	82-83	$C_{15}H_{24}N_2O_4S_2$	7·76	8·14
45	C_4H_9O	H	Cyclohexyl	135-6	$C_{17}H_{26}N_2O_4S_2$	7·78	7·56

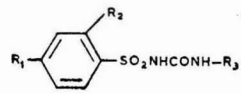
(150 ml.). The solution, after extraction with ethylene dichloride (30 ml.) to remove the unreacted isothiocyanate, was then carefully acidified under cooling to obtain the thiourea derivative which was collected and crystallized from ethanol yielding colourless needles; m.p. 144-5°C.; yield 7·5 g. (84 per cent).

N-(4-Methoxybenzenesulphonyl)-N'-cyclohexylurea — N-(4-Methoxybenzenesulphonyl)-N'-cyclohexylthiourea (5·0 g.) was dissolved in aqueous sodium bicarbonate (3 per cent; 100 ml.), and hydrogen peroxide (15 per cent; 25 ml.) added dropwise over a period of 15 min. with stirring, and maintaining the temperature of the reaction mixture around 35°C. Stirring was continued for 10 min. more, the resulting mass cooled and filtered. The filtrate was acidified with hydrochloric acid to get a white crystalline precipitate which was collected, washed with water, and crystallized from ethanol; m.p. 182-3°C.; yield 3·33 g. (73 per cent).

All the benzenesulphonylthioureas and the ureas prepared by the above method are listed in Tables 1 and 2 respectively.

Testing for hypoglycaemic activity in normal fasting rabbits — Normal healthy rabbits bred at the Institute, weighing about 2 kg., were used. The animals

TABLE 2 — N-N'-DISUBSTITUTED SULPHONYLUREAS



Code No.	R_1	R_2	R_3	M.P. °C.	Mol. formula	Nitrogen, % Found	Nitrogen, % Reqd	Hypoglycaemic activity in rabbits§ (*dose* 50 *mg./kg.*) 3 hr	Hypoglycaemic activity in rabbits§ (*dose* 50 *mg./kg.*) 5 hr
R-94	H	H	Isopropyl	119-20	$C_{10}H_{14}N_2O_3S$	11·52	11·57	21·6	26·3
R-131	H	H	*n*-Butyl	131-3†	$C_{11}H_{16}N_2O_3S$	—	—	19·4	19·3
Butamide	CH_3	H	do	—	—	—	—	25·1	27·9
R-296	CH_3	H	Isobutyl	169-71‡	$C_{12}H_{18}N_2O_3S$	—	—	25·1	23·0
R-286	H	CH_3	Methyl	207-9	$C_9H_{12}N_2O_3S$	12·55	12·28	12·1	3·1
R-284	H	CH_3	Ethyl	201-2	$C_{10}H_{14}N_2O_3S$	11·60	11·57	nil	5·6
R-285	H	CH_3	*n*-Propyl	162-3	$C_{11}H_{16}N_2O_3S$	11·21	10·93	Rise in blood sugar 6-11%	
R-280	H	CH_3	Isopropyl	179-80	$C_{11}H_{16}N_2O_3S$	11·09	10·93	10·2	24·6
R-282	H	CH_3	Isobutyl	153-4	$C_{12}H_{18}N_2O_3S$	10·37	10·37	Rise in blood sugar 7-11%	
R-281	H	CH_3	Cyclohexyl	171-2	$C_{14}H_{20}N_2O_3S$	10·00	9·46	14·0	22·9
R-291	Cl	H	Ethyl	141-2‡	$C_9H_{11}ClN_2O_3S$	—	—	19·8	23·1
R-292	Cl	H	Allyl	176-8	$C_{10}H_{11}ClN_2O_3S$	10·43	10·20	Rise in blood sugar 6-9%	
Chloro-propamide	Cl	H	*n*-Propyl	128-30‡	—	—	—	12·6	11·2
R-293	Cl	H	Isobutyl	167-9	$C_{11}H_{15}ClN_2O_3S$	10·04	9·63	11·3	17·8
R-276	CH_3O	H	Isopropyl	139-40	$C_{11}H_{16}N_2O_4S$	9·59	10·23	20·9	18·3
R-275	CH_3O	H	Isobutyl	140-2‡	$C_{12}H_{18}N_2O_4S$	—	—	24·0	16·1
R-274	CH_3O	H	*n*-Butyl	119-21	$C_{12}H_{18}N_2O_4S$	—	—	15·2	19·0
R-277	CH_3O	H	Cyclohexyl	182-3‡	$C_{14}H_{20}N_2O_4S$	—	—	19·5	24·5
R-244	C_2H_5O	H	Isopropyl	169-70	$C_{12}H_{18}N_2O_4S$	9·81	9·79	nil	nil
R-253	C_2H_5O	H	*n*-Butyl	162-4	$C_{12}H_{20}N_2O_4S$	—	—	nil	16·6
R-441	C_3H_7O	H	Allyl	128-9	$C_{13}H_{18}N_2O_4S$	8·95	9·39	nil	nil
R-436	C_3H_7O	H	*n*-Propyl	148-50	$C_{13}H_{20}N_2O_4S$	9·11	9·33	nil	nil
R-437	C_3H_7O	H	Isopropyl	140-1	$C_{13}H_{20}N_2O_4S$	9·51	9·33	nil	nil
R-438	C_3H_7O	H	*n*-Butyl	142-3	$C_{14}H_{22}N_2O_4S$	9·26	8·91	nil	nil
R-439	C_3H_7O	H	Isobutyl	163-4	$C_{14}H_{22}N_2O_4S$	8·93	8·91	nil	nil
R-440	C_3H_7O	H	Cyclohexyl	177-8	$C_{16}H_{24}N_2O_4S$	8·13	8·23	nil	nil
R-427*	C_4H_9O	H	Methyl	144-5	$C_{12}H_{18}N_2O_4S$	9·99	9·79	8·3	16·1
R-514	C_4H_9O	H	Allyl	115-17	$C_{14}H_{20}N_2O_4S$	8·88	8·98	nil	nil
R-430	C_4H_9O	H	Isopropyl	133-4	$C_{14}H_{22}N_2O_4S$	8·43	8·91	2·1	10·4
R-432	C_4H_9O	H	Isobutyl	147-9	$C_{15}H_{24}N_2O_4S$	8·01	8·53	nil	nil
R-433	C_4H_9O	H	Cyclohexyl	159	$C_{17}H_{26}N_2O_4S$	7·51	7·90	8·3	1·0

*Reported by Delacoux *et al.*[15].
†Reported by Marshall and Sigal[9].
‡Reported by Ruschig *et al.*[10].
§Per cent reduction in blood sugar in fasting normal rabbits with a single oral dose after intervals of 3 and 5 hr (figures represent the average of blood sugar values of six animals).

were fasted for 20-24 hr prior to the experiment and no food was given during the course of experiment but water was allowed to be taken all the time. Fasting blood was collected from the marginal ear vein. A suspension of the drug (50 mg./kg.) in gum acacia was administered orally in a single dose by the stomach tube, followed by the administration of plain gum acacia solution by the same stomach tube to transfer the drug quantitatively into the animal. Blood samples were collected at 1·5, 3 and 5 hr, and in some cases 7, 24 and 48 hr after the administration of the drug. Suitable controls were also run without the drug. Blood sugar estimations were made by Somogyi's method using Nelson's reagent[7]. The hypoglycaemic activities of the compounds are given in Tables 3 and 4.

Testing in rats and guinea-pigs — Healthy adult albino rats (weighing 200-250 g.) and guinea-pigs (weighing 450-500 g.) bred and reared at the Institute were used. They were fasted for 20-24 hr prior to experiment and after taking a fasting sample of blood, the drug was administered in a single dose orally by special bent needles. Blood samples were collected and blood sugar determined as already described in the case of rabbits. Blood was collected from the tail vein in rats and by cutting a small portion of the toe in guinea-pigs. The results obtained are given in Table 3.

TABLE 3 — ORAL HYPOGLYCAEMIC ACTIVITY OF SULPHONYLUREAS

(*A single dose of* 10 *mg./kg. administered*)

Animals	Drug	% blood sugar reduction (mean) at different intervals after drug administration					
		1·5 hr	3 hr	5 hr	7 hr	24 hr	48 hr
Rabbits (12 animals)	Tolbutamide	9·4±3·13	21·0±2·15	19·8±2·04	18·8±2·14	6·1±1·88	—
	Chloropropamide	8·9±1·46	10·9±1·01	12·9±1·46	7·9±0·92	1·2±0·53	—
	R-94	15·7±2·36	19·7±1·83	27·9±1·89	21·6±2·59	18·4±2·65	—
	R-131	11·9±1·80	10·4±1·09	9·7±1·05	6·7±0·46	1·5±0·51	—
	R-277	6·6±1·65	10·9±2·07	12·7±1·75	—	13·6±2·17	9·6±2·54
Rabbits (6 animals)	Control (change from F.B.S,)	−2·2±1·78	nil±1·31	+2·6±1·36	+4·3±2·21	+0·6±1·43	−0·2±1·67
Rats (16 animals)	Tolbutamide	5·6±0·96	15·9±1·55	5·7±0·75	3·7±0·71	1·9±0·66	—
	Chloropropamide	16·3±2·62	21·4±1·88	31·0±2·45	28·7±2·36	12·6±2·01	3·7±0·89
	R-94	25·4±1·37	28·2±1·38	37·6±2·31	31·3±1·14	22·9±2·30	16·1±1·76
	R-131	16·6±2·19	21·8±1·92	22·4±2·24	23·5±2·15	18·1±1·18	14·2±1·60
	R-277	12·6±1·31	24·7±1·96	27·1±1·54	—	20·6±1·65	9·3±1·30
Rats (6 animals)	Control (change from F.B.S.)	−2·4±1·87	−2·8±1·71	+0·2±2·56	+0·2±2·56	+0·6±2·32	0·9±3·16
Guinea-pigs (6 animals)	Tolbutamide	6·4±2·21	22·9±1·34	16·5±2·35	12·8±1·41	12·6±1·07	—
	Chloropropamide	16·9±3·14	22·6±2·37	27·2±1·31	22·5±2·59	11·6±1·62	—
	R-94	13·2±2·97	24·1±1·27	24·1±2·84	24·1±2·59	22·9±3·50	—
	Control (change from F.B.S.)	−3·0±1·28	−1·8±1·24	nil±1·04	+2·3±1·08	+2·0±0·92	—

F.B.S.= fasting blood sugar.

TABLE 4 — ORAL HYPOGLYCAEMIC ACTIVITY OF R-94, R-280, R-281 AND TOLBUTAMIDE IN FASTING RABBITS

(*Single oral dose of* 25 *mg./kg. administered; values are mean of* 6 *animals*)

Drug	% reduction of blood sugar at different intervals after administration of drug				
	1·5 hr	3 hr	5 hr	7 hr	24 hr
R-94	10·2	22·5	25·3	27·7	—
R-280	22·0	25·7	20·0	19·0	16·10
R-281	21·6	26·7	23·0	16·2	13·60
Tolbutamide	8·4	20·8	22·1	17·1	—

Results and discussion

Out of several sulphonylureas studied, 15 compounds showed definite hypoglycaemic action in rabbits at a dose level of 50 mg./kg. equivalent to or in some cases better than tolbutamide. Three compounds were found to be hyperglycaemic. The remaining compounds showed no significant action at the dose level studied.

Three compounds, viz. N-benzenesulphonyl-N′-isopropylurea (R-94), N-benzenesulphonyl-N′-*n*-butylurea (R-131) and N-*p*-methoxybenzenesulphonyl-N′-cyclohexylurea (R-277), were found to possess definite and prolonged hypoglycaemic action, maintaining relatively steady hypoglycaemic levels over an extended period of time beyond 24 hr. The preparation of these three compounds by different method has been described previously[8-10] but the hypoglycaemic activity reports have not been available.

McLamore *et al.*[1], who have earlier studied the effects of various substituents in the benzenesulphonylureas on the hypoglycaemic activity, noted that the introduction of a substituent such as amino, methyl or halogen in the benzene ring gives the most active compounds, and that *p*-chloro group prolongs the duration of action. Cardani *et al.*[11] have mentioned that compounds with no substituents in the benzene ring are much less potent. We have found that, amongst the compounds with no substituent in benzene ring but having different alkyl substituents in the terminal nitrogen, two (R-94 and R-131) possess pronounced hypoglycaemic activity in rabbits. These two compounds were studied further in rats (R-94 in guinea-pigs also) with smaller dosage of 10 mg./kg. and both were found to have a definite and prolonged action.

Among the compounds having a methyl group in the *para* position of the benzene ring (analogues of tolbutamide) only one compound having isobutyl radical at the terminal nitrogen showed good activity. Shortening the alkyl chain reduced the activity considerably.

It is reported that *ortho*-substituted benzenesulphonylurea derivatives do not show good hypoglycaemic activity[1]; however, two derivatives with a methyl group in *ortho* position studied by us possessed good activity. Further studies with these compounds at lower dose level have shown them to

have steady and well-maintained action over a long duration of time (Table 4).

Chloro substitution in the *para* position of the benzene ring generally increased the activity. Four compounds with chlorine in *para* position of benzene ring studied in rabbits indicated good activity.

Most of the N-*p*-methoxybenzenesulphonyl-N'-alkyl or cyclohexyl ureas, have shown good hypoglycaemic action. McColl *et al.*[12] had reported *p*-methoxy analogue of tolbutamide (R-274) to be as active as tolbutamide and to be less toxic, while Popova[13] reported the same compound to be inactive. We have found this substance to be fairly active but the maximum tolerated oral dose in mice was found to be lower than that of tolbutamide. In this series, however, N-*p*-methoxybenzenesulphonyl-N'-cyclohexylurea (R-277) has given a definite and prolonged hypoglycaemic activity. Other *p*-alkoxy derivatives tested did not show any substantial activity except R-427 in the butoxy series which showed slight activity.

The compound R-94 has been found to be a potent hypoglycaemic agent. When orally administered, a definite effect has been obtained in rabbits with as low a dose as 5 mg./kg. At the same dose level, tolbutamide or chloropropamide did not show any significant hypoglycaemic activity in rabbits. The results of the tests carried out with three different doses of R-94, viz. 5, 10 and 15 mg./kg. respectively, are given graphically in Fig. 1. In normal fasting animals, no significant reduction in blood sugar has been observed. These tests were carried out in the same group of animals with an interval of 6-7 days between different doses. As seen from Fig. 1, the response increases with increasing dose up to 3-5 hr which can be termed as immediate hypoglycaemic phase. At the end of 5-7 hr period, which can be termed as persistent hypoglycaemic phase, a noticeable effect greater in magnitude could be observed with 10 and 15 mg./kg. doses, as compared to 5 mg./kg. dose. But the effect between 10 and 15 mg./kg. are not noticeably different. Optimum response could be obtained in rabbits at a dose level of 10 mg./kg. at the 5-7 hr period (i.e. 25·5 to 36·6 per cent reduction with an average 29·9 per cent in six animals). Further increase in dose did not elicit any noticeable increase in the magnitude of response. The tests were repeated with different doses of tolbutamide using different groups of rabbits. The results are graphically presented in Fig. 2. When compared with dosages of equivalent potency, the dosage of R-94 is found to be lower than that of tolbutamide. With tolbutamide the maximum response is obtained at a dose level of 50 mg./kg. at about

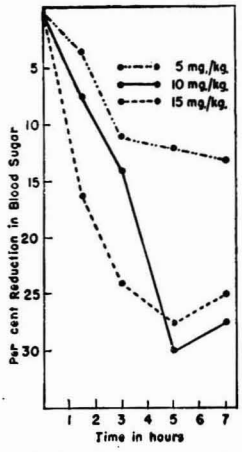


Fig. 1 — Dose response of R-94 in rabbits

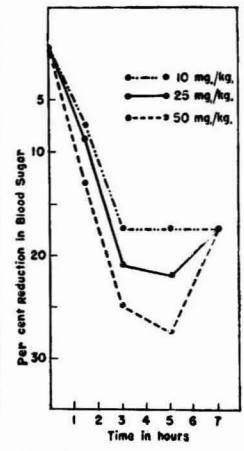


Fig. 2 — Dose response of tolbutamide in rabbits

5 hr period. These differences, whether due to the absorbabilities of the drugs or to the difference in the mechanism of action, are yet to be elucidated.

Cross over tests — Since R-94 and R-131 were found to possess good hypoglycaemic activity, these have been compared with two other well-known oral hypoglycaemic agents, viz. tolbutamide and chloropropamide. There are several methods for studying the comparative effect or the activities of two similarly active compounds. If it is possible to measure the effects of each preparation, on each animal, experimental design known as cross over test may be used. In this method, the advantage is that the effect of any difference between the general level of response at the two stages of the test may be balanced. Using this method cross over tests of R-94 and R-131 with chloropropamide and tolbutamide have been carried out in rabbits and rats. Oral dose of a drug used was 10 mg./kg. and the interval between the cross tests was about a week. The results of the tests with these two drugs are given with statistical analysis in Tables 5-8.

In rabbits at a single oral dose of 10 mg./kg. tolbutamide and R-94 showed significant reduction of blood sugar up to 7 hr and slight reduction of 5·7 per cent (mean of six results) after 24 hr. The difference in the hypoglycaemic effect of the two drugs was not significant up to 7 hr but at 24 hr the difference as judged by t value becomes significant. Similar results have been obtained in rabbits with R-94 and chloropropamide.

Similar tests were carried out in rats with R-94 and R-131 and chloropropamide. Since tolbutamide at a dose level of 10 mg./kg. has given considerably less response in rats, the cross over test

TABLE 5 — COMPARATIVE HYPOGLYCAEMIC ACTIVITY OF R-94 AND TOLBUTAMIDE: CROSS OVER TESTS WITH RABBITS

(*No. of animals used*, 6; *dose of drugs*, 10 *mg./kg.*)

Exp. period *hr*	Reduction in blood sugar, %						Diff. of means	Diff. of S.E.	*t* df=5
	R-94			Tolbutamide					
	Mean	S.D. (±)	S.E. (±)	Mean	S.D. (±)	S.E. (±)			
1·5	11·1	6·78	2·77	11·2	12·34	5·04	−0·1	2·900	0·010*
3	15·4	3·95	1·61	23·5	8·41	3·43	−8·1	3·256	2·494*
5	31·7	4·07	1·66	22·3	8·11	3·31	+9·4	4·020	2·358*
7	29·2	1·75	0·71	20·4	9·12	3·73	+8·8	3·624	2·423*
24	26·4	4·07	1·66	5·7	6·52	2·66	+20·7	2·750	7·539†

*Difference in the percentage reduction of blood sugar is not significant at 5 per cent level.
†Difference in the percentage reduction of blood sugar is significant at 0·1 per cent level in favour of R-94.

TABLE 6 — COMPARATIVE HYPOGLYCAEMIC ACTIVITY OF R-94 AND CHLOROPROPAMIDE: CROSS OVER TESTS WITH RABBITS

(*No. of animals used*, 6; *dose of drugs*, 10 *mg./kg.*)

Exp. period *hr*	Reduction in blood sugar, %						Diff. of means	Diff. of S.E.	*t* df=5
	R-94			Chloropropamide					
	Mean	S.D. (±)	S.E. (±)	Mean	S.D. (±)	S.E. (±)			
1·5	20·25	7·19	2·94	9·97	6·29	2·57	+10·60	4·509	2·350*
3	24·12	5·17	2·11	10·20	4·25	1·73	+13·80	3·581	3·855†
5	24·07	6·53	2·66	11·20	4·71	1·92	+12·80	4·360	2·947*
7	13·95	5·85	2·40	8·40	2·99	1·22	+5·60	3·164	1·756*
24	10·45	4·02	1·64	0·60	1·43	0·58	+9·90	1·647	5·980‡
30	5·17	6·20	2·53	—	—	—	—	—	—
48	0·97	2·37	0·97	—	—	—	—	—	—

*Difference in the percentage reduction in blood sugar is not significant at 5 per cent level.
†Difference in the percentage reduction in blood sugar is significant at 5 per cent level.
‡Difference in the percentage reduction in blood sugar is significant at 1 per cent level.

with this drug has been omitted or, in other words, the cross over tests have been restricted to a known drug, viz. chloropropamide, which gives a good response in this species. The results obtained were similar to those obtained with rabbits. Both the drugs R-94 and R-131 have shown a steady and definite fall in the blood sugar and this reduction was well maintained up to 48 hr.

Toxicity studies — The maximum tolerated oral dose in rats for the three most promising compounds, viz. R-94, R-131 and R-277, was found to be 4000 mg./kg., 2000 mg./kg. and 4000 mg./kg. respectively as against tolbutamide with 2500 mg./kg. LD_{50} in mice by subcutaneous injection for R-94, R-131 and tolbutamide were found to be 2050 mg./kg., 530 mg./kg. and 980 mg./kg. respectively. For chloropropamide, the LD_{50} in mice is reported to be 780 mg./kg.[14]. Preliminary oral acute toxicity studies in mice indicate R-275, R-276, R-280 and R-281 to be better tolerated than tolbutamide.

Growth studies — In these experiments the effect of tolbutamide and R-94 were compared on the gain in weight in weanling rats, weighing about 40-45 g. Each group consisted of 10 rats and the animals were daily fed orally the drugs at dose level of 100 mg./kg. for a period of 8 weeks. The animals were weighed every week and also observed for any untoward symptoms of toxicity such as weakness, diarrhoea, or vomiting and tremors. They were fed with the stock diet of this Institute. The animals maintained good progress throughout the study and the gain in weight during the first two weeks was practically the same in all the three groups, viz. the control, tolbutamide treated and R-94 treated. Between the second and fourth week, animals in the control group gained weight at a slightly faster rate than the two drug treated groups. However, at the end of eighth week no noticeable difference in weight gain of animals in all the three groups was observed. The average weight at the end of eighth week for the control group was 137 g. while for the tolbutamide treated and R-94 treated animals this average was 130 and 133 g. respectively. It may be mentioned here that chloropropamide at the dose level used has been reported to have a depressant effect[14] on the rats.

TABLE 7 — COMPARATIVE HYPOGLYCAEMIC ACTIVITY OF R-94 AND CHLOROPROPAMIDE: CROSS OVER TESTS WITH RATS

(*No. of animals used*, 8; *dose of drugs*, 10 *mg./kg.*)

Exp. period *hr*	Reduction in blood sugar, %						Diff. of means	Diff. of S.E.	*t* df=6
	R-94			Chloropropamide					
	Mean	S.D. (±)	S.E. (±)	Mean	S.D. (±)	S.E. (±)			
1·5	23·8	5·59	2·11	16·3	11·03	4·17	+7·5	5·336	1·416*
3	26·7	7·05	2·66	19·8	8·84	3·34	+6·9	5·454	1·265*
5	32·6	13·63	5·15	29·1	9·66	3·65	+3·5	8·104	0·434*
7	30·2	5·57	2·11	32·5	8·89	3·36	−2·3	4·008	0·567*
24	21·6	11·32	4·28	11·8	3·73	1·41	+9·8	5·271	1·851*
48	13·3	9·62	3·64	4·2	4·12	1·66	+9·1	3·932	2·303*

*Difference not significant at 5 per cent level.

TABLE 8 — COMPARATIVE HYPOGLYCAEMIC ACTIVITY OF R-131 AND CHLOROPROPAMIDE: CROSS OVER TESTS WITH RATS

(*No. of animals used*, 8; *dose of drugs*, 10 *mg./kg.*)

Exp. period *hr*	Reduction in blood sugar, %						Diff. of means	Diff. of S.E.	*t* df=7
	R-131			Chloropropamide					
	Mean	S.D. (±)	S.E. (±)	Mean	S.D. (±)	S.E. (±)			
1·5	18·0	10·42	3·68	21·8	9·18	3·24	−3·75	5·620	0·667*
3	18·5	9·91	3·50	21·9	6·82	2·41	−3·38	4·358	0·774*
5	19·7	9·08	3·21	25·4	7·16	2·53	−5·68	3·015	1·882*
7	18·7	8·67	3·07	18·9	11·16	3·95	−0·20	5·209	0·048*
24	17·8	6·20	2·19	10·6	11·53	4·08	+7·20	1·400	1·400*
48	14·2	8·31	2·94	8·6	8·42	2·98	+5·60	3·685	1·510*

*Difference not significant at 5 per cent level.

From the results of our studies, as well as the structure activity studies carried out by other workers[1] earlier, it seems that it is not possible to anticipate the hypoglycaemic activity of the substituted phenyl sulphonylureas from the nature and the location of the substituents in the benzene ring or the length and character of the alkyl chain attached to the terminal nitrogen.

Preliminary clinical trials of R-94, R-131 and R-277 in patients with diabetes mellitus are at present being carried out by Dr J. C. Patel at K. E. M. Hospital, Parel, Bombay 12. The results of clinical trials would be published elsewhere.

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Pharmacognostical Study of *Cissampelos pareira* Linn.

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The macroscopical and the microscopical details of the stems and roots of *Cissampelos pareira* have been presented. The stem and the root have some common morphological features such as the presence of a more or less continuous sclerenchymatous layer, wedges of xylem tissue and broad medullary rays. The root shows anomalous growth structure and the outstanding features are the presence of several converging arms of lignified xylem having broad medullary rays between them and uni- to triseriate medullary rays within the xylem strands and small patches of phloem over the xylem strands. The structure of the xylem of the rootlet differs from that of the main root.

THE family *Menispermaceae* is reputed for yielding plants with curariform activity[1-5]. The true *Pareira brava* is reported to be derived from *Chondodendron tomentosum*, which grows in tropical Africa and yields tube curare[2,3]. *Cissampelos pareira* roots have also appeared in commerce as false *Pareira brava*[2-5]. It is a dioecious, lofty, perennial climber attaining heights up to 15 ft, and growing throughout the tropical and subtropical parts of India, extending to warm regions of Asia, East Africa and America up to an altitude of 6000 ft.

The roots of *C. pareira* (Hindi: *Parha* or *Parhi*) are the most valued part of the plant[1-3]. It has been held in great esteem in the Ayurvedic system of medicine and has been recommended as a substitute for the costly imported drug, tubocurarine[2]. The drug has been used in the indigenous systems of medicine in the treatment of dysentery, skin eruptions, cancer, burning and itching. It also removes intestinal worms, cures enlarged spleen and is useful in heart trouble, asthma and intestinal tuberculosis.

Due to its great importance the drug has been the subject of several investigations[5-10]. Wigger[6] isolated an amorphous alkaloid, pelosine, from the roots of South American species of *C. pareira*. Scholtz[7] reported that pelosine is identical with bebeerine. Bhattacharji *et al.*[8-10], working on the Indian species, reported the presence of two crystalline alkaloids, hyatin, m.p. 303°C. (decomp.), and hyatinin, m.p. 163°C., quercitol, m.p. 235°C., and a sterol, m.p. 140-1°C. However, they were not able to isolate pelosine reported by Wigger and Scholtz in the American species. Roy *et al.*[11] isolated from the roots 0·718 per cent total alkaloids, fatty matter, a bitter resin, waxy substance, sterol and traces of an essential oil. The curariform activities of hyatin methiodide and hyatin methochloride have been studied by Roy *et al.*[11] and Pradhan *et al.*[12], and compared with that of *d*-tubocurarine chloride by Pradhan and De[13].

The pharmacognostical aspect of the drug has, however, not been properly investigated. The previous observations[14,15] are meagre and inadequate, and at places contradictory (*vide infra* Discussion). It was, therefore, deemed necessary to undertake a thorough and detailed study, with a view to bringing out all the diagnostic features of the drug. As a considerable proportion of stem occurs usually in the market samples of the drug, the present investigation includes the study of the stem as well, so that the genuine drug (root) may easily be distinguished from the adulterant (stem).

Experimental procedure

Fresh portions of stem and root of *C. pareira* were collected from the Ayurvedic Garden of the Banaras Hindu University. Samples of the drug, sold under the name of *Parha* or *Parhi*, were also obtained from the local drug market. Sections by a razor and a hand microtome were taken and the cell contents and cell-wall structure studied according to the procedure described elsewhere[16,17].

Macroscopical features

Stem (Plate I, Figs. 1-4) — The stem is a climber, striate, wiry, green and softly pubescent when young, 0·4-0·6 cm. in diameter at the base where the bark is pale brownish and fairly smooth; the wood is porous, which, with 60 per cent H_2SO_4, turns green.

Root (Plate I, Fig. 5 and Plate III, Fig. 15) — The roots are perennial varying in length and thickness, being 2·8-14·5 cm. long and 2-13 mm. in diameter. They are cylindrical in shape, which often become

tortuous in old roots. In thinner roots, the cork is smooth and brownish, while in the older pieces, the surface is rough and at places rugged due to the presence of transverse wrinkles, cracks or fissures. A smoothened transverse surface reveals a dark line (representing the ring of stone cells — *vide infra*) and the xylem dissected by radiating arms of xylem, having plenty of starch in the intervening soft white parenchyma. The root in the upper region is more woody. Fracture is short and splintery. Fresh roots are devoid of any odour, but stored samples develop a faint aroma. Taste is bitter.

Microscopical features

Stem (Plate II, Figs. 6-10) — The young stem shows a single-layered epidermis many cells of which are elongated to form bicellular covering trichomes, measuring 180-**290**-455 μ in length (Fig. 6). The epidermis does not rupture for a long time even after

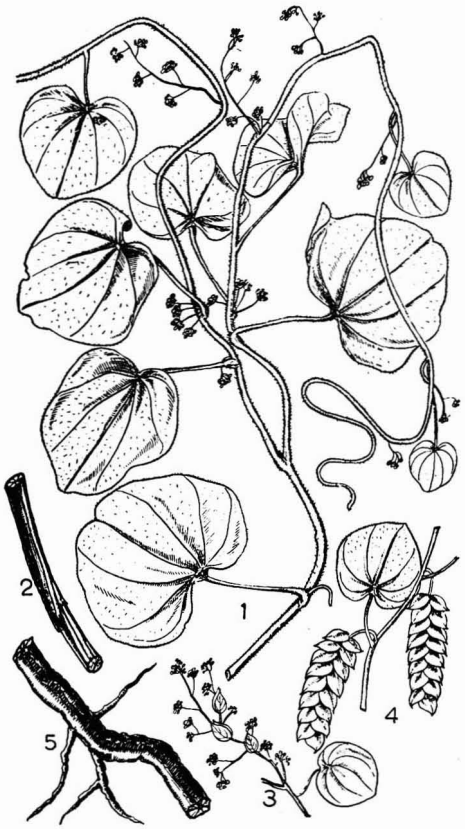

Plate I — Macroscopic characters of stem, leaves, flowers and root [Fig. 1: A part of the young stem with branches, leaves and male flowers. Fig. 2: Mature stem from basal part. Fig. 3: Cyme of male flower. Fig. 4: Raceme of female flowers. Fig. 5: Root. All ×$\frac{1}{3}$]

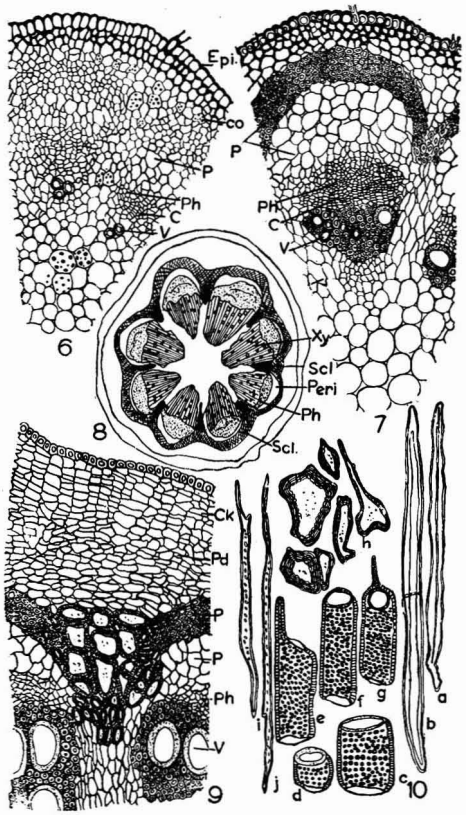

Plate II — Microscopic characters of young and mature stem [Fig. 6: T.s. of young stem × 56. Fig. 7: T.s. of slightly mature stem × 56. Fig. 8: T.s. of mature stem (diagrammatic) × 4. Fig. 9: Details of a portion of Fig. 8 × 56. Fig. 10: Isolated elements. *a* and *b*, pericyclic fibres; *c-g*, tracheae; *h*, stone cells; *i* and *j*, xylem fibres; *Epi*, epidermis; *Co*, collenchyma; *P*, pericycle; *Ph*, phloem; *C*, cambium; *V*, vessel; *Xy*, xylem; *Scl*, sclerenchyma; *Ck*, cork; *Pd*, phellcderm]

the formation of cork, the cork cambium first originating in the second layer of the cortex. The cortex is represented by only 3-5 layers of cells, which are slightly collenchymatous in the outer two layers. Endodermis is absent. The vascular bundles are arranged in a ring and each vascular bundle is capped by a pericycle, the cells of which gradually become thick walled and lignified in the outer 5-7 layers and form sclerenchymatous fibres (Fig. 7), measuring 155-**525**-915 μ by 16-**18**-22 μ. Some of the pericyclic fibres are also septate, possessing one or two septa. The inner 3-5 layers of pericycle remain parenchymatous, the individual cells measuring 20-**48**-60 μ by 28-**44**-50 μ. These parenchymatous cells, in advanced stages, often get disintegrated and form cavities or lacunae.

There are usually 5-7 vascular bundles separated by 4-7 cells wide medullary rays. The cells of the medullary rays are parenchymatous and radially elongated, but with age, the outer ray cells and adjoining cortical cells become sclerosed, forming moderately thick-walled stone cells (50-**80**-100 μ by 27-**30**-36 μ). In older stems, therefore, a continuous and composite pericycle is formed of sclerenchymatous fibres, stone cells, and parenchyma surrounding the vascular bundles (Figs. 8 and 9). During further growth, new vascular bundles arise by the meristematic activity of medullary ray cells and the cortical cells form sclerenchymatous layer over these newly formed bundles. The number of vascular bundles thus increases up to eleven in mature stems although eight vascular bundles are more common (Fig. 8). Occasionally, the new vascular bundle fuses with the neighbouring old bundle with the pericycle capping over the entire vascular bundle. The phloem consists of sieve tubes, companion cells and phloem parenchyma. The xylem is composed of tracheae, tracheids, fibres and xylem parenchyma. The vessels are drum-shaped with simple and bordered pits and measure 50-**140**-180 μ by 28-**44**-50 μ. The xylem fibres measure 270-**480**-976 μ by 14-**16**-18 μ.

In the old stems, some of the xylem vessels are filled with a brownish content and the primary phloem of the vascular bundles often gets crushed. The central pith is composed of parenchymatous cells which are more or less isodiametric.

Root (Plate III, Figs. 11-19) — A transverse section of the young root shows a single-layered epidermis followed by a parenchymatous cortex of 4-6 layers of isodiametric cells. The endodermis is distinct, showing casparian dots and measure 12-**28**-45 μ by 8-**20**-24 μ (Fig. 11). Some of the endodermal cells contain raphides of calcium oxalate, 8-**10**-20 μ in length. Below the endodermis is a continuous layer of thin-walled pericyclic cells. The primary xylem exhibits a diarch or tetrarch structure; the former is more common in the main root, while the latter in daughter roots.

The secondary growth starts in the normal way and in the daughter roots a central cylinder of wood surrounded by a ring of phloem is formed (Fig. 14). In the main root, however, there is irregular growth of the vascular tissue; the central xylem plate (Fig. 12) develops more below the two phloem strands and ultimately bulges outward forming two projecting arms at right angles to it. The phloem tissues in such roots are also pushed out along with the developing arms and are distinctly visible at their ends.

During the development of secondary phloem a few peripheral cells become lignified, and give rise to phloem fibres (Figs. 12 and 13). New fibres and stone cells are later added by the pericycle and medullary ray cells. A composite and continuous ring of sclerenchyma, partly of phloem origin and partly of pericyclic and ray origin, is thus formed in a mature root (Figs. 15 and 16). The stone cells are spherical, oval or elongated (Fig. 19) and also irregular in shape. The stone cells show simple pits on the walls and measure 32-**46**-58 μ by 22-**26**-40 μ.

There are a few more new vascular strands formed by the meristematic activity of the ground parenchyma of the medullary ray cells below the pericycle. With further growth a number of radiating strips (usually 8-12) of xylem and phloem are to be observed in the mature root (Fig. 15). These strips vary in their radial length, some reaching even the centre of the xylem, while the newly formed bundles are small and are seen at the periphery.

Medullary rays are of two types. Uni- to triseriate rays occur within the radially running xylem arms and wider rays between these radially running arms of xylem. Occasionally the broad medullary rays are divided by formation of new radiating xylem strands. Part of the xylem parenchyma gets delignified and thus adds to the medullary rays. In a tangential section the narrow rays measure 30-**44**-**68**-105 μ in width and 190-**250**-**985**-1465 μ in length (Fig. 17), while the wider ones measure 480-**560**-**860**-960 μ in width and 120-**400**-**1900**-3140 μ in length. A few cells of the medullary ray become thick walled and lignified. Some of the ray cells also show reticulate thickening. The cells contain plenty of starch grains.

The cork cells are rectangular and 4-7 cells thick, measuring 34-**52**-61 μ by 10-**18**-26 μ. The phelloderm cells are oval to tangentially elongated 1-3 cells thick and often show a radial arrangement. The phloem consists of phloem parenchyma, sieve tubes and companion cells, while xylem consists of tracheae, tracheids, fibres and xylem parenchyma. The vessels are mostly drum-shaped and show straight or oblique articulations with perforation rims. They measure 80-**145**-180 μ by 24-**32**-48 μ. The tracheids have tapering ends. Both the vessels and tracheids have simple and bordered pits on their walls. The tracheids measure 186-**215**-248 μ by 18-**20**-24 μ. The fibres are 220-**264**-340 μ in length and 14-**16**-18 μ in breadth with tapering ends and show simple pits on the wall. The lignified xylem parenchyma is represented by thick-walled, pitted, rectangular cells.

Ash content

The total and acid-insoluble ash contents were determined according to the standard procedure[17]. The total ash content varies from 6·58 to 6·80 per cent and the acid-insoluble ash from 0·98 to 1·13 per cent.

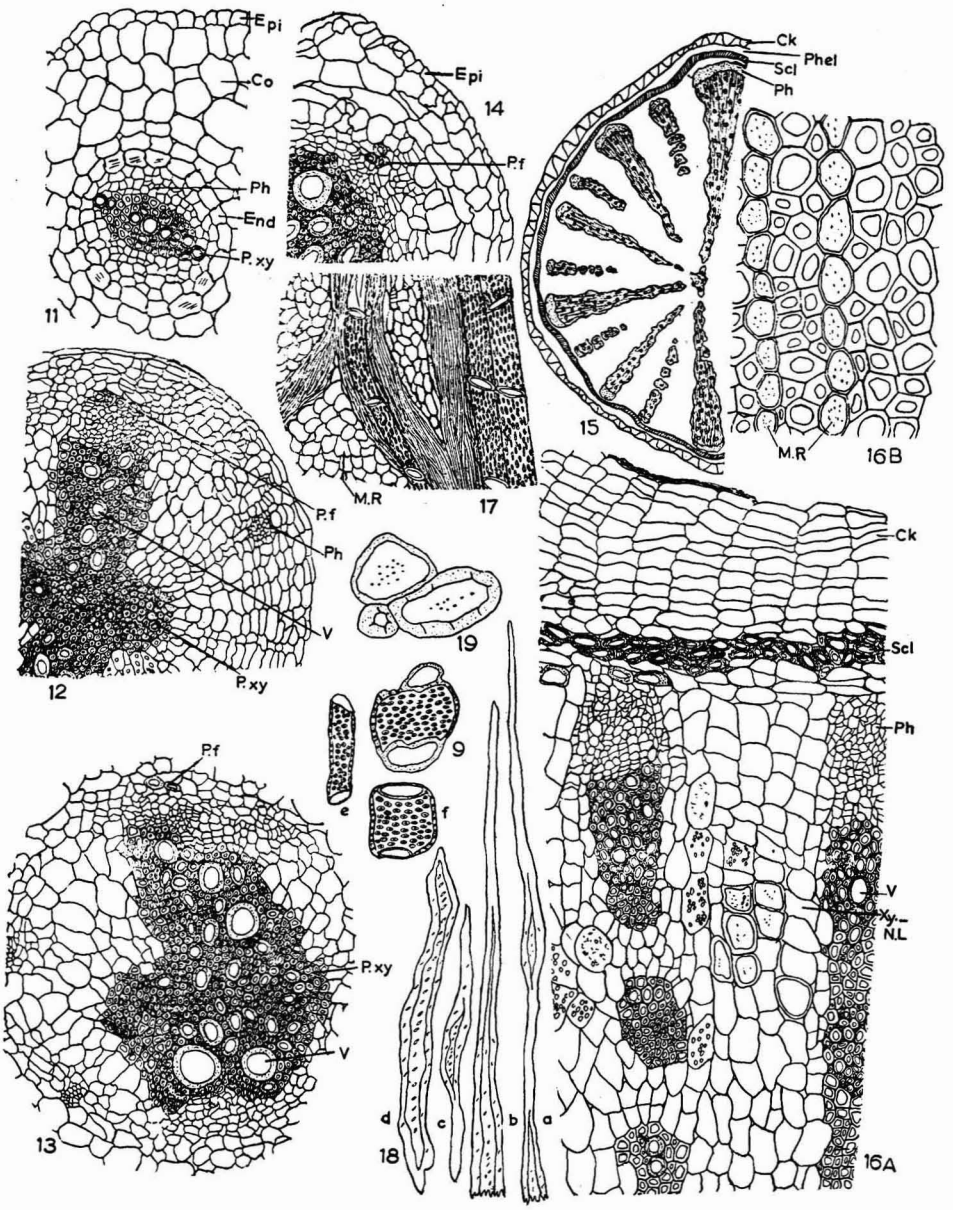

Plate III — Microscopic characters of young and mature root [Fig. 11: T.s. of young root × 65. Fig. 12: T.s. of root showing the bulging xylem arms × 75. Fig. 13: T.s. of root showing irregular growth of the central xylem plate × 75. Fig. 14: T.s. of a thin daughter root × 75. Fig. 15: T.s. of mature root × 5. Fig. 16A: Details of a portion of Fig. 15 × 75. Fig. 16B: T.s. of xylem showing narrow medullary rays. Fig. 17: Tangential section of root from xylem region × 75. Fig. 18: Xylem elements — *a* and *b*, fibre ends, *c*, fibre tracheid; *e-g*, vessels × 80. Fig. 19: Stone cells of root × 90. *Epi*, epidermis; *Co*, cortex; *Ph*, phloem; *End*, endodermis; *P.xy*, protoxylem; *P.f*, phloem fibre; *V*, vessel; *Xy*, xylem; *Ck*, cork; *Phel*, phelloderm; *Scl*, sclerenchyma; *Xy.N.L*, non-lignified xylem]

Microchemical tests

Alkaloid is present in all parts of the stem and root excepting the cork. Acicular crystals of calcium oxalate are present in the cortex and pith of the stem and in the delignified xylem parenchyma of the root. Starch is abundant in all parts of the stem and xylem except the lignified xylem tissue. Starch grains are mostly spherical and simple (**4-12-18-26** μ in diameter), a few are cup-shaped with small beaks. The compound grains are of 3-5 components.

Discussion

From the foregoing description it will be seen that the stem of *C. pareira* can easily be distinguished from its root by the presence of a well-developed pith in the former, while the root possesses a central woody parenchymatous core. However, there are certain structural features common to both, as for instance, the possession of a continuous layer of sclerenchyma formed in the stem by the pericycle and the medullary rays, and in the root by the pericycle, the secondary phloem and the medullary rays. On this point, the findings of Datta and Mukerji[14] are contradictory and erroneous and not in agreement with observations recorded herein, inasmuch as they have mentioned the presence of a sclerenchymatous ring in the secondary phloem of the root, and yet maintained that bast fibres are not present in the secondary bast. Further, they have shown a continuous ring of phloem in their diagram with the sclerenchymatous ring outside the secondary phloem, while the fact is that the phloem occurs only as strands (not a continuous ring), being separated by medullary rays, and the sclerenchymatous ring is formed jointly, as pointed above, by the phloem, the medullary rays and the pericycle. Again, such statements as " the cells of phelloderm are like the cortical cells " when actually no cortex exists after the formation of the phelloderm, and " the xylem is composed of broad medullary rays " are highly confusing since the rays are of two types: narrow uni- to triseriate medullary rays and wide multiseriate medullary rays which also include often part of delignified xylem parenchyma. More or less similar confusion has been made regarding the medullary rays and other structures by Aiyar *et al.*[15] who observed that no elements of mechanical tissue are associated with phloem proper, that stone cells contained crystals and that the ridges in the outline correspond in number to the wedge-like masses of wood. These facts are incorrect in the light of the observations presented above. It may as well be suggested that the I.P.C.[18] monograph, which mentions the same erroneous statements of Datta and Mukerji[14], should be modified in the light of the present observations.

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Nutritive Value of Field Bean (*Dolichos lablab*): Part I—*In vitro* Digestibility of Raw & Autoclaved Beans

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***In vitro* digestibility experiments indicate that raw or autoclaved field beans are less digestible by pepsin and trypsin, than casein. Autoclaving the beans does not show any effect on their digestibility by pepsin. However, the digestibility of raw beans by (i) trypsin and (ii) pepsin followed by trypsin is increased by autoclaving. The increase in the digestibility is maximum when the beans are autoclaved for 5 min. at 15 lb./sq. in. pressure and is minimum when it is autoclaved for 4 hr. Microbiological estimations of amino acids indicate that field beans are deficient in almost all essential amino acids as compared to casein and methionine is the most limiting amino acid.**

FIELD bean is one of the most commonly consumed legume in all parts of India and by all sections of the population and hence it was considered of interest to study its nutritive value. Dhonde and Sohonie[1,2] have isolated the proteins of field bean and have studied the *in vitro* digestibility of the two predominant proteins of field bean. The present work relates to the study of the *in vitro* digestibility of raw field bean and the effect of autoclaving on its digestibility. The digestibility of the field bean has been studied by subjecting the raw and autoclaved legume to enzymic hydrolysis and the extent of hydrolysis is followed by determining the rate of release of (i) total soluble nitrogen, (ii) amino acid nitrogen and (iii) individual amino acids.

Experimental procedure

A preliminary experiment was carried out in which casein, raw field bean and field bean autoclaved for 5 min., 1, 2, 3 and 4 hr at 15 lb. pressure were subjected to enzymic hydrolysis with (i) pepsin, (ii) trypsin and (iii) pepsin followed by trypsin. Casein was used as the standard reference protein. The hydrolysis was carried out as described later. The digestibility of the field bean was studied by determining the rate of release of total soluble nitrogen. The digestibility of casein is more than that of either raw or autoclaved field bean. Autoclaving the field bean does not seem to affect the peptic digestibility. However, autoclaving has a marked effect on tryptic hydrolysis of field bean which is indicated by increased tryptic digestibility of autoclaved field bean. Maximum increase is observed when the pulse is autoclaved for 5 min. and is minimum when the pulse is autoclaved for 4 hr. Hence, in the subsequent and more detailed studies, field bean subjected to autoclaving for 5 min. and 4 hr was used.

Peptic digestion — Casein (1 g.) or 5 g. each raw and autoclaved field bean flour were taken in different flasks and suspended in 100 ml. of 0·05*M* HCl-KCl buffer[3] (*p*H 1·8). Pepsin solution (0·05 per cent; 10 ml.) was added to each flask. Aliquots (10 ml.) of the reaction mixtures were immediately taken out and treated with 15 ml. of 5 per cent trichloracetic acid (TCA) to separate undigested protein. The flasks were incubated at 37°C. and aliquots (10 ml.) of the reaction mixtures were pipetted out after 2, 4 and 24 hr and treated with TCA solution as before. The filtrates obtained by separating the precipitates were used for the estimations of total soluble nitrogen by the micro-Kjeldahl method.

Tryptic digestion — Casein (1 g.) or raw and autoclaved field bean flour (5 g.) were taken in different flasks and suspended in 100 ml. of 0·05*M* Sorensen's phosphate buffer[4] (*p*H 7·6) and trypsin (Merck) solution (0·05 per cent; 10 ml.) was added to each flask. Aliquots (10 ml.) were taken out at the start of the reaction and after 2, 4 and 24 hr and treated with TCA solution (5 per cent; 15 ml.). Aliquots (10 ml.) of the filtrates obtained by separating the precipitates were used for the estimations of total soluble nitrogen by the micro-Kjeldahl method.

Peptic digestion followed by tryptic digestion — Casein (2·5 g.) or 12·5 g. each of raw and autoclaved

TABLE 1 — RATE OF RELEASE OF SOLUBLE NITROGEN BY ENZYMIC HYDROLYSIS OF RAW AND AUTOCLAVED BEANS

Enzyme	Release of soluble nitrogen (*g./100 g. protein*) from							
	Casein	Raw beans	Beans autoclaved for					
			5 min.	30 min.	1 hr	2 hr	3 hr	4 hr
Pepsin	4·83	1·63	1·59	1·50	1·51	1·51	1·54	1·57
Trypsin	8·88	1·51	3·41	3·39	3·42	3·42	2·36	2·21
Pepsin followed by trypsin	10·16	2·02	5·03	5·01	5·00	4·92	4·68	3·90

field bean flour were taken in different flasks and suspended in 250 ml. of 0·05*M* HCl-KCl buffer (*p*H 1·8) and pepsin solution (0·05 per cent; 25 ml.) was added to each flask. Hydrolysis was allowed to proceed for 2 hr, aliquots (50 ml.) of the reaction mixtures being drawn at the start of reaction and after 2 hr. The *p*H of the residual solutions was adjusted to 7·6 and trypsin solution (0·05 per cent; 15 ml.) added to each flask, and hydrolysis carried out for a further period of 24 hr. Aliquots of the reaction mixtures (50 ml.) were drawn after 4, 6 and 24 hr of reaction. The aliquots treated with TCA and the filtrates obtained after separation of precipitate were used for the estimation of soluble nitrogen by the micro-Kjeldahl method. However, to determine the rate of release of amino acid nitrogen and individual essential amino acids, aliquots of the reaction mixtures taken out at different intervals were adjusted to *p*H 4·5, heated in a boiling water bath for 30 min., filtered and neutralized. Suitable aliquots of the neutralized filtrate were used for the estimations of (i) amino acid nitrogen by Sorensen's formol titration method[5] and (ii) essential amino acids by microbiological method[6].

To study the total amino acid composition, casein, raw field bean and field bean autoclaved for 5 min. and 4 hr at 15 lb. pressure were hydrolysed with acid (with alkali for the estimation of tyrosine and tryptophan) in an autoclave for 8-10 hr. The acid, alkali and enzyme hydrolysates were used for the determination of amino acids by microbiological methods. The technique used for culturing and harvesting the organisms were the same as described by Barton-Wright[6]. The basal media employed in the several determinations were prepared according to Greenhurt *et al.*[7], Schweigert *et al.*[8], Barton-Wright[9] and Barton-Wright and Curtis[10].

Results and discussion

The results of these estimations are reported in Tables 1-3 and Figs. 1-3.. Figs. 1-3 indicate that raw or autoclaved field bean is less digestible by

TABLE 2 — AMINO ACIDS CONTENT OF CASEIN, RAW AND AUTOCLAVED BEANS

Amino acids	Amino acids (*g./100 g. protein*)			
	Casein	Raw beans	Beans autoclaved for	
			5 min.	4 hr
Arginine	3·71	8·78	9·22	8·84
Threonine	3·61	3·32	4·77	3·55
Leucine	11·12	9·95	10·03	9·94
Isoleucine	6·82	5·33	4·92	5·02
Valine	9·13	5·50	5·55	5·87
Histidine	3·48	2·50	3·46	2·59
Phenylalanine	5·03	3·94	4·92	3·87
Lysine	8·15	7·44	7·48	7·24
Methionine	3·72	0·83	1·24	0·85
Tryptophan	1·34	0·52	0·77	0·52
Cystine	0·65	0·62	0·62	0·62
Tyrosine	5·20	5·01	5·01	5·01

proteolytic enzymes as compared to casein. Autoclaving does not improve the digestibility of raw field bean by pepsin. In the case of tryptic digestion or peptic digestion followed by tryptic digestion, the digestibility is improved by autoclaving, the improvement being maximum in the case of field bean autoclaved for 5 min. and minimum with that autoclaved for 4 hr. The digestibility of soyabean has also been found to be improved by autoclaving for 4 min. and lowered by autoclaving it for 4 hr[11].

Field bean is deficient in all the essential amino acids, except arginine, as compared to casein (Table 2). It is highly deficient in methionine and in tryptophan and valine, methionine being the most limiting amino acid. Autoclaving for 5 min. increases the amounts of threonine, histidine, methionine and tryptophan, while the amounts of these amino acids are decreased when the bean is autoclaved for 4 hr. Viswanatha and De[12] have reported that N-S complexes of sulphur containing amino acids of soyabeans are rendered easily hydrolysable by autoclaving. Drastic heat treatment leads to the destruction of a number of amino acids of soyabean[13-15]. Similar changes might occur in the case of field bean also, due to which the

TABLE 3 — RATE OF RELEASE OF AMINO ACIDS BY PEPTIC HYDROLYSIS FOLLOWED BY TRYPTIC HYDROLYSIS OF RAW AND AUTOCLAVED BEANS

Amino acids	Release of amino acids (%) from															
	Casein				Raw beans				Beans autoclaved for 5 min.				Beans autoclaved for 4 hr			
Incubation period, hr:	2	4	6	24	2	4	6	24	2	4	6	24	2	4	6	24
Arginine	39	57	82	87	34	49	60	63	34	49	67	74	34	46	57	60
Threonine	4	21	41	51	3	20	32	38	4	20	32	40	4	20	30	39
Leucine	16	33	40	74	13	25	38	53	16	30	43	65	13	28	41	58
Isoleucine	0	7	15	30	0	8	15	29	0	8	15	27	0	7	15	29
Valine	7	17	42	62	6	11	30	50	7	12	30	50	6	12	30	49
Histidine	8	11	12	25	4	9	10	19	6	10	11	22	4	8	10	19
Phenylalanine	15	42	52	71	5	32	42	51	9	36	50	60	5	34	46	54
Lysine	7	10	22	35	4	8	12	14	7	12	17	19	5	10	10	15
Methionine	15	45	50	90	8	11	30	42	8	15	35	46	8	11	31	35
Tryptophan	9	35	45	63	7	25	35	43	7	28	35	44	7	28	35	39
Cystine	15	29	51	63	10	25	40	60	12	27	48	62	10	22	37	60
Tyrosine	10	40	50	69	10	35	40	60	10	40	48	64	10	40	45	64

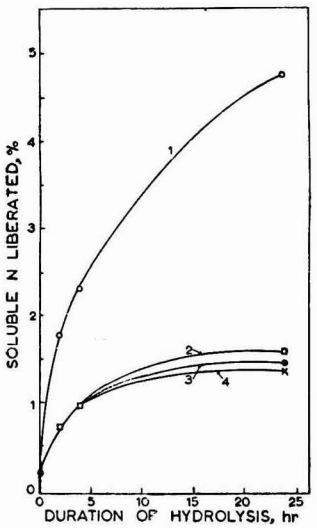

Fig. 1 — Liberation of soluble nitrogen by peptic hydrolysis of (1) casein, (2) raw beans, (3) beans autoclaved for 5 min. and (4) beans autoclaved for 4 hr

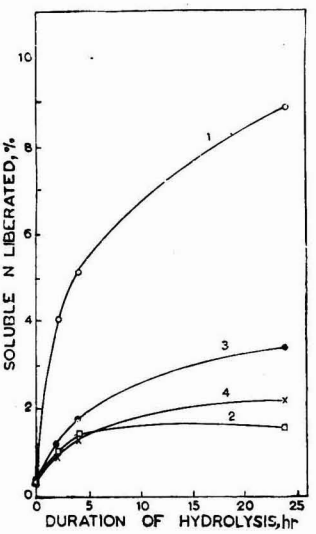

Fig. 2 — Liberation of soluble nitrogen by tryptic hydrolysis of (1) casein, (2) raw beans, (3) beans autoclaved for 5 min. and (4) beans autoclaved for 4 hr

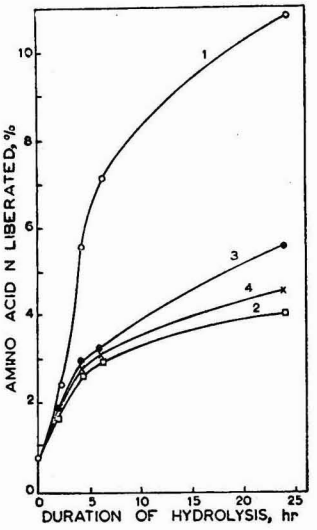

Fig. 3 — Liberation of soluble nitrogen by peptic hydrolysis followed by tryptic hydrolysis of (1) casein, (2) raw beans, (3) beans autoclaved for 5 min. and (4) beans autoclaved for 4 hr

amounts of essential amino acids are increased by autoclaving the pulse for 5 min. and decreased by autoclaving for 4 hr.

The rate of release of amino acids from raw field bean by enzymic digestion is low as compared to that of casein. The rate of release of arginine, leucine and lysine is increased by autoclaving the field bean for 5 min. and that of arginine, leucine, lysine, phenylalanine and methionine is decreased by autoclaving for 4 hr. Ambe and Sohonie[16] have established the presence of heat labile trypsin inhibitor in raw field bean. The low digestibility of raw field bean by trypsin may perhaps be attributed to the presence of trypsin inhibitor which retards the action of proteolytic enzymes. Increase in the digestibility of the field bean by autoclaving it for 5 min. may be due to the destruction of such inhibitor. The digestibility of field bean is decreased by autoclaving for 4 hr, probably due to the changes brought about in the protein linkages as described by Eldred and Rodney[17].

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New Local Anaesthetics: Part II—Derivatives of 5-Morpholino-(& 5-piperidino)-acetylamino-2-arylimino-3-aryl-4-thiazolidones

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Ten new 5-morpholino- and ten new 5-piperidino-acetylamino-2-arylimino-3-aryl-4-thiazolidones having different aryl substituents have been synthesized and screened for local anaesthetic activity. The 5-morpholino-acetylimino thiazolidones are more active than the corresponding 5-piperidino-acetylamino thiazolidones. Among morpholino derivatives, the tolyl-substituted thiazolidones are more potent than others. Substitution in the *p*-position has been found to confer greater activity on the compound than substitution elsewhere of a higher aromatic nucleus. The hydrochloride of 5-morpholino-acetylamino-2-*p*-tolylimino-3-*p*-tolyl-4-thiazolidone has been found to be the most active of the compounds synthesized.

IN the first part of this series[1], it was shown that several 5-diethylamino-acetylamino-2-arylimino-3-aryl-4-thiazolidones exhibit local anaesthetic activity and that the activity of the compounds was influenced by the various substituents and their positions. It was considered of interest to find out whether substitution of 5-diethylamino moiety by 5-morpholino or 5-piperdino moiety in the thiazolidones will yield more active compounds.

5-Amino-2-arylimino-3-aryl-4-thiazolidones, the starting materials in this work, were synthesized as described earlier[1]. These compounds, on condensation with chloroacetyl chloride[1,2] and subsequent treatment with morpholine and piperidine, gave the required 3-morpholino-acetylamino thiazolidones and 5-piperidino-acetylamino thiazolidones respectively in good yields. These were converted into hydrochlorides in the usual manner.

The compounds were screened according to the procedure adopted by Bulbring and Wajda[3]. It is interesting to note that the local anaesthetic activity of the compounds varies with variations within the general structure of these compounds and that the activity of the 5-morpholino-acetylamino thiazolidones is greater than that of the corresponding 5-piperidino-acetylamino thiazolidones. In morpholino derivatives, the tolyl-substituted thiazolidones are more potent that other morpholino derivatives. In general, *para* substitution in the benzene ring confers more activity on the

TABLE 1 — PHYSICAL CHARACTERISTICS OF 5-MORPHOLINO-(AND 5-PIPERIDINO)-ACETYLAMINO-2-ARYLIMINO-3-ARYL-4-THIAZOLIDONES

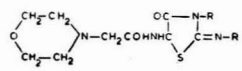
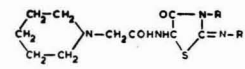
Sl No.	R	Yield %	M.P. °C.	Mol. formula	Sulphur, % Found	Sulphur, % Reqd	M.P. of hydrochlorides °C.
				5-MORPHOLINO DERIVATIVES			
							
1	Phenyl-	69	170	$C_{21}H_{22}N_4O_3S$	7·77	7·80	177
2	*p*-Chlorophenyl-	56	174	$C_{21}H_{20}Cl_2N_4O_3S$	6·65	6·68	180
3	*o*-Tolyl-	57	155	$C_{23}H_{26}N_4O_3S$	7·28	7·30	152
4	*m*-Tolyl-	62	159	$C_{23}H_{26}N_4O_3S$	7·26	7·30	155
5	*p*-Tolyl	80	115	$C_{23}H_{26}N_4O_3S$	7·29	7·30	130
6	*o*-Anisyl-	62	185	$C_{23}H_{26}N_4O_5S$	6·76	6·81	200
7	*p*-Anisyl-	65	118	$C_{23}H_{26}N_4O_5S$	6·75	6·81	110
8	*o*-Phenetyl-	57	115	$C_{25}H_{30}N_4O_5S$	6·40	6·43	110
9	α-Naphthyl-	78	165	$C_{29}H_{26}N_4O_3S$	6·25	6·27	176
10	β-Naphthyl-	71	145	$C_{29}H_{26}N_4O_3S$	6·28	6·27	108
				5-PIPERIDINO DERIVATIVES			
							
11	Phenyl-	81	178	$C_{22}H_{24}N_4O_2S$	7·80	7·84	173
12	*p*-Chlorophenyl-	69	170	$C_{22}H_{22}Cl_2N_4O_2S$	6·67	6·71	167
13	*o*-Tolyl-	74	162	$C_{24}H_{28}N_4O_2S$	7·31	7·33	158
14	*m*-Tolyl-	61	86	$C_{24}H_{28}N_4O_2S$	7·30	7·33	225
15	*p*-Tolyl-	70	230	$C_{24}H_{28}N_4O_2S$	7·30	7·33	164
16	*o*-Anisyl-	60	193	$C_{24}H_{28}N_4O_4S$	6·80	6·84	189
17	*p*-Anisyl-	65	118	$C_{24}H_{28}N_4O_4S$	6·81	6·84	180
18	*o*-Phenetyl-	53	124	$C_{26}H_{32}N_4O_4S$	6·40	6·45	161
19	α-Naphthyl-	76	150	$C_{30}H_{28}N_4O_2S$	6·29	6·30	147
20	β-Naphthyl-	79	148	$C_{30}H_{28}N_4O_2S$	6·28	6·30	159

TABLE 2 — LOCAL ANAESTHETIC ACTIVITY OF HYDROCHLORIDES OF 5-MORPHOLINO-(AND 5-PIPERIDINO)-ACETYLAMINO-2-ARYLAMINO-3-ARYL-4-THIAZOLIDONES

	Onset of anaesthesia (*min.*) with administration of anaesthetic† in hydrochloric acid of strength					
	0·05*N*	0·1*N*	0·2*N*		0·05*N*	0·1*N*	0·2*N*
	5-MORPHOLINO DERIVATIVES				5-PIPERIDINO DERIVATIVES		
Sl No.*				Sl No.*			
1	5·0	7·0	8·0	11	8·0	10·0	10·5
2	4·0	6·0	7·0	12	9·5	10·0	11·0
3	4·0	9·0	10·5	13	8·0	9·0	9·5
4	6·5	6·5	7·0	14	7·5	9·0	9·0
5	2·0	3·5	5·0	15	9·0	10·5	11·0
6	7·0	9·0	10·5	16	6·0	7·5	8·5
7	4·0	8·5	9·0	17	9·5	11·0	12·5
8	5·0	11·0	12·5	18	7·0	8·5	9·0
9	9·0	10·5	11·0	19	7·0	8·0	8·5
10	4·0	11·0	13·0	20	6·0	7·5	9·0
Procaine hydrochloride	5·0	8·0	10·0	Procaine hydrochloride	5·0	8·0	10·0

*Sl Nos. correspond to Sl Nos. of compounds in Table 1.
†Concentration of anaesthetic, 0·2 per cent.

compound than the presence of a higher aromatic nucleus. Piperidino derivatives are, in general, the least active.

Experimental procedure

5-Morpholino-acetylamino-2-phenylimino-3-phenyl-4-thiazolidone — A mixture of 5-chloroacetylamino thiazolidone (3 g.) dissolved in absolute alcohol (50 ml.) and morpholine (1·2 ml.) was refluxed on a water bath for 4-5 hr. The excess of alcohol and morpholine was recovered by distillation and the residue washed first with aqueous sodium bicarbonate and finally with distilled water. The product was crystallized from benzene.

The 5-morpholino-acetylamino-2-arylimino-3-aryl-4-thiazolidones and 5-piperidino-acetylamino-2-arylimino-3-aryl-4-thiazolidones synthesized are listed in Table 1.

The hydrochlorides were prepared by dissolving the above bases each (2 g.) in dry ether and in some cases in benzene (20 ml.) and saturating the solution with dry HCl gas. Ether was decanted off and the semi-solid mass was recovered from absolute alcohol. The melting points of the hydrochlorides are also included in Table 1.

Plexus anaesthesia in frog[1] — A 0·2 per cent solution of the hydrochlorides was used and the time of onset of anaesthesia was recorded and compared with that of procaine hydrochloride as the standard. The results are given in Table 2.

Acknowledgement

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Short Communications

Transamination in *Azotobacter chroococcum*

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Out of 15 amino acids tested for transamination in *A. chroococcum*, the highest activity is recorded in the case of aspartate-glutamate system. Aspartate-alanine and glutamate-alanine systems are rather weak. The properties of the four transaminase systems studied in cell-free preparation show that the optimum *p*H for the reaction lies between 8·0 and 10·0. Transaminase activity of cell-free preparation is found to be consistently higher than that of intact resting cells. Acetone treatment partially inactivates aspartate-glutamate and alanine-glutamate systems but not the other two systems. Alanine-glutamate, valine-glutamate and isoleucine-glutamate systems are almost completely inhibited by 1 *mM* concentration of hydroxylamine, while aspartate-glutamate system is less sensitive to it. Dinitrophenol and ethylene diamine tetra acetate in 1 *mM* concentration have no significant action on these transaminase systems.

THE presence of transaminase systems in animal, plant and bacteria was reported by various workers[1-5]. But the transaminase systems present in soil organisms such as *Azotobacter chroococcum* have not been studied. Only the occurrence of *aspartic-glutamic* system has been reported in *Azotobacter vinelandii*[3-6]. The present paper relates to the study of different transaminase systems of *A. chroococcum*.

A. chroococcum used was isolated from hill soils of neutral to slightly alkaline reaction. The nitrogen fixing capacity of the organism was about 10-12 mg. of nitrogen per gram of sugar consumed.

The above strain obtained from the Microbiology Section of this Institute was grown on Burk's nitrogen-free agar medium. Cell suspensions in 0·02*M* phosphate buffer (*p*H 8·0) for enzymatic studies were made by harvesting 3 days' growth on Burk's agar with 0·005*M* phosphate buffer. Acetone-dried cells were prepared by dispersing freshly harvested cells in cold acetone, centrifuging at 1500 r.p.m. for 15 min. at 0°C. and drying the acetone-free sediment *in vacuo*. Cell-free extracts were prepared by alternate freezing and thawing of freshly harvested cells followed by grinding with alumina at 0°C. The clear supernatant obtained by centrifuging this extract was used as the cell-free preparation.

TABLE 1 — TRANSAMINASE ACTIVITY IN RESTING CELL SUSPENSION

[*Activity expressed as μg. of glutamic acid-N formed/mg. of dry bacteria. Reaction mixture (final concentrations)*: 1 *ml. of cells* (15-15·5 *mg.*) *in* 0·003*M phosphate buffer* (*p*H 8·0); 20 *μmoles of α-ketoglutaric acid*; 20 *μmoles of amino acid*; 0·5 *ml. of* 0·033*M phosphate buffer*, *p*H 8·0; *temp.* 37°*C.*; *incubation period*, 2 *hr*]

Amino acids added to α-ketoglutaric acid	Glutamic acid-N *μg./mg. dry bacteria*
Aspartic acid	4·44
DL-Alanine	2·73
Phenylalanine	1·57
Tyrosine	1·15
Methionine	2·00
Isoleucine	3·45
Serine	0·85
Arginine	2·12
Lysine	1·52
Histidine	1·70
Proline	2·01
L-Leucine	1·79
Tryptophan	0·18
Valine	4·20
Glycine	0·85

Transamination reaction was followed anaerobically in Thunberg tubes using 0·5 ml. of 0·2*M* phosphate buffer (*p*H 8·0), 0·2 ml. of 0·1*M* α-ketoglutarate or sodium pyruvate, 0·2 ml. of 0·1*M* of the amino acid, 0·5 ml. of enzyme preparation and 10 μg. of pyridoxal phosphate in a total volume of 3·0 ml. The reaction was terminated by addition of 10 per cent trichloroacetic acid, and glutamic acid or alanine present in the clear supernatant was assayed by paper chromatography.

The rate of transamination with 15 amino acids as amino group donors by resting cells of the organism is given in Table 1. Aspartic acid was found to be most active, valine following very closely and then isoleucine. Similar observations have also been reported with other organisms[4,5,7]. Aspartate-alanine and glutamate-alanine systems were rather weak in this organism. Negative results have been reported in the case of *Bacillus subtilis*[8]. Synthesis of alanine from pyruvate and amino acids has been reported to proceed very slowly, even after 8 hr incubation, in *Vibrio cholerae*[9].

The rate of transaminase activity with cell-free preparation for four amino acids as amino donors is given in Fig. 1. Aspartate-glutamate system recorded the highest activity as in the case of *Salmonella typhosa*[10]. In case of intact cells, unlike the cell-free system, the reactions of all the four systems were linear and valine-glutamate system showed the highest activity, aspartate-glutamate system following very closely.

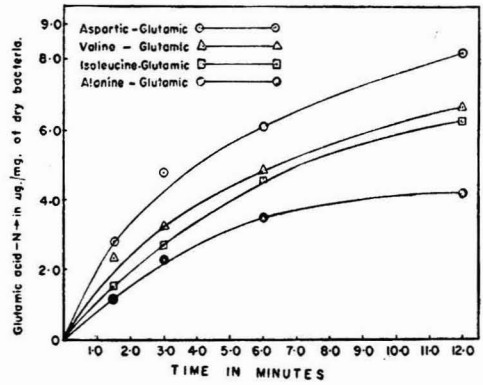

Fig. 1 — Time-activity curve of transaminase systems in cell-free preparation

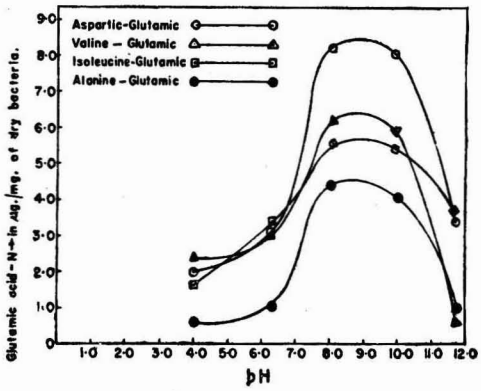

Fig. 2 — *p*H curve of transaminase systems in cell-free preparation

The *p*H curves for the four transaminase systems given in Fig. 2 show that the optimum *p*H of the reactions is in the region of 8·0-10·0.

The activity of cell-free preparation was found to be consistently higher than that of intact cells as evident from the results in Tables 2 and 3. This is apparently due to permeability factors. Lower activities were reported with L-alanine and DL-aspartic acid in cell-free extracts of *Pasteurella pestis* as compared to the activities showed by the intact cells[11].

Acetone treatment largely inactivated aspartate-glutamate and alanine-glutamate systems, while valine-glutamate and isoleucine-glutamate systems were not at all affected (Table 3).

The effects of different inhibitors on these four transaminase systems were also studied. Dinitrophenol and ethylene diamine tetra acetate in 1 *mM* concentration were found to have no significant

TABLE 2 — COMPARATIVE TRANSAMINASE ACTIVITY OF INTACT CELLS AND CELL EXTRACT

(*Reaction period*, 2 *hr*; *temp.*, 37°*C.*; *p*H, 8·0)

Dry wt equiv. *mg.*	Glutamic acid-N, *µg.*	
	Fresh cells	Cell extract
3·0	15·75	19·42
6·0	25·56	43·50
12·0	35·40	75·46
18·0	48·06	89·74

TABLE 3 — TRANSAMINASE ACTIVITIES IN DIFFERENT PREPARATIONS

[*Substrate concentration* (*final*), 0·033*M*; *temp.*, 37°*C.*; *p*H, 8·0; *reaction period*, 2 *hr*]

Transaminase system	Glutamic acid-N *µg./mg. of dry bacteria*			
	Fresh intact cells	Acetone-dried cells	Fresh cell extract	Acetone-dried cell extract
Aspartic-glutamic	4·4	2·0	7·5	0·4
Valine-glutamic	4·2	4·3	6·7	2·1
Isoleucine-glutamic	3·5	3·4	6·0	1·4
Alanine-glutamic	2·7	1·3	5·1	0·5

action on transaminase activity, whereas hydroxylamine in 1 *mM* concentration produced 55·5 per cent inhibition with aspartic acid, 85·6 per cent with alanine, 94·4 per cent with valine and 97·6 per cent with isoleucine.

The results of inhibition studies and comparative inactivation by cold acetone treatment appear to indicate the presence of different transaminases in this organism.

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Physico-chemical Studies on Indigenous Seed Proteins: Part VI — Amino Acid Composition of Red Gram (*Cajanus indicus*) Meal & Globulin Fractions

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The amino acid composition of red gram meal as well as its three globulin fractions show the absence of histidine and low amounts of methionine and cystine. The main globulin fraction (I) contains most of the essential amino acids in good proportion except lysine, threonine and tryptophan. Lysine is present to a greater extent in fraction II, threonine in fraction III, and tryptophan both in fractions II and III.

IN our earlier studies on the physico-chemical aspects of seed proteins, data on the solubility and electrophoretic characterization of red gram meal proteins were reported[1]. Studies on the isolation, fractionation and electrophoretic characterization of its globulins were given in a subsequent publication[2].

The present paper deals with the results of our investigations on the amino acid composition of the red gram meal and its globulin fractions.

The red gram globulin fractions were obtained by dilution and fractional precipitation method described previously[2]. The hydrolysates of the meal and globulins were prepared according to the method of Block *et al.*[3]. Nitrogen in the protein and the hydrolysates was estimated by the micro-Kjeldahl procedure.

The amino acid composition of the hydrolysates was determined by circular paper chromatographic technique employing the following solvent systems: (1) butanol-acetic acid-water (4: 1: 5); (2) phenol-isopropyl alcohol-water (14: 1: 5) in the atmosphere of 0·3 per cent ammonia; (3) butanol-water-ethanol (20: 4·5: 1); and (4) butanol-acetic acid-water (20: 3·5: 2·5). The details of this method have been described by Nath *et al.*[4]. Tryptophan was estimated chemically by the procedure of Graham *et al.*[5].

The amino acid composition of the red gram meal and of the three globulin fractions is given in Table 1. A number of reports[6-8] are available in literature on the amino acid composition of red gram meal. However, the amino acid values reported by Bagchi *et al.*[6] for this meal have been given for comparison since they have been obtained by paper chromatography. The values obtained

TABLE 1 — AMINO ACID COMPOSITION OF RED GRAM MEAL AND THE DIFFERENT FRACTIONS*

(*g. per* 100 *g. substance*)

Amino acid	F I	F II	F III	Meal	Meal†
Alanine	9·25	5·46	2·48	0·96	0·86
Arginine	1·78	1·31	1·14	1·74	1·63
Aspartic acid	6·93	3·24	2·04	2·92	2·70
Cystine	3·02	Traces	Traces	0·89	0·81
Glutamic acid	12·43	8·57	5·88	3·44	3·97
Glycine	3·42	4·32	5·24	0·86	0·75
Histidine	—	—	—	—	—
Isoleucine	7·37	4·66	3·72	1·86	2·92
Leucine	9·07	3·47	2·93	1·09	—
Lysine	1·81	4·46	1·62	1·84	2·20
Methionine	0·42	Traces	Traces	0·20	—
Phenylalanine	7·35	0·46	0·32	1·52	1·75
Proline	6·23	1·56	1·00	2·21	1·17
Serine	4·75	4·00	4·14	1·72	1·31
Threonine	2·08	1·89	4·93	1·75	1·30
Tryptophan	0·07	0·37	0·32	0·51	0·25
Tyrosine	1·23	0·76	0·43	0·96	0·73
Valine	4·20	0·99	0·49	1·39	1·10

*Values are mean of ten observations.
†Values reported by Bagchi *et al.*[6].

for most of the amino acids in the present study agree fairly well with those reported by Bagchi *et al.* Values for proline and tryptophan, however, show marked differences. In agreement with the results of these workers, histidine was found to be absent in the meal.

Very often a detailed amino acid analysis only of the seed meals is made and, in some cases, of its chief globulin fraction. That the assessment of the nutritive value of the whole protein attempted from such data is fallacious has been pointed out by Jones in the case of peanut proteins[9]. He showed that its main globulin, arachin, is deficient both in tryptophan and methionine while the minor globulin, conarachin, contains relatively higher amounts of these acids. As a result, nutritional supplementation occurs only when both globulins are fed. Consequently, the necessity of studying the amino acid composition of all the fractions of any protein before inference could be drawn on its nutritive value is of significance.

As is generally observed in the case of leguminous seeds, the red gram meal and its various fractions are deficient in methionine, the maximum concentration of which occurs in fraction I. Cystine content is comparatively low and is also mainly present in fraction I. Among the basic amino acids, histidine is absent in the meal and the fractions. While the lysine content of fractions I and III is almost the same as that of the meal, it is present in much higher amounts in fraction II. The acidic amino acids, aspartic and glutamic acids are present to varying extents in the different fractions, both being highest in fraction I.

Among other essential amino acids, phenylalanine, tyrosine and valine are present in highest amounts in fraction I, threonine in fraction III and tryptophan both in fractions II and III. Leucine and isoleucine are present largely in fraction I and in considerable amounts in the other two fractions also.

Though the major globulin fraction I contains most of the essential amino acids, it is, however, deficient in tryptophan, threonine and lysine and consequently nutritional supplementation can occur only when the other fractions are also present.

The authors' thanks are due to the Council of Scientific & Industrial Research, New Delhi, for financial assistance.

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Effect of Osmotic Pressure on the Synthesis of Fat by *Penicillium aurantiobrunneum*

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The influence of osmotic pressure produced by high concentrations (1-4 per cent) of sodium, potassium, magnesium and calcium chlorides in the growth medium on the synthesis of fat by *P. aurantiobrunneum* has been investigated. Sodium and potassium chlorides at 2 per cent level induce maximum synthesis of fat whereas in the case of magnesium and calcium chlorides it occurs at 1 per cent level. A medium containing 1 per cent each of sodium and calcium chlorides gives the highest yield (30 per cent on the weight of felt) of fat.

HALDEN[1] reported that the osmotic pressure of the medium exerts a profound influence on the synthesis of fat by moulds and fungi. Heide[2] observed an increase of fat content in *Endomyces vernalis*

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TABLE 1 — EFFECT OF VARYING THE CONCENTRATIONS OF THE SALTS USED INDIVIDUALLY IN THE MEDIUM ON MYCELIAL WEIGHT AND FAT FORMATION

(*Vol. of culture medium*, 50 *ml.*; *incubation period*, 16 *days*; *incubation temp.*, 25°C.)

Wt of salt g./100 ml.	Wt of felt g.	Wt of fat g.	Wt of sugar consumed g.	Fat % of felt	Fat coeff.	Economic coeff.
			POTASSIUM CHLORIDE			
0·000	3·3390 ±0·109	0·3530	14·90	10·57	2·37	22·40
0·500	3·2104 ±0·072	0·3620	14·63	11·27	2·47	21·95
1·000	3·0962 ±0·161	0·3750	14·71	12·11	2·54	21·40
1·500	2·8546 ±0·055	0·4090	14·62	14·32	2·85	19·52
2·000	2·7552 ±0·047	0·4120	14·57	14·95	2·75	18·91
3·000	1·2340 ±0·197	0·3830	13·79	31·03	2·77	8·94
4·000	1·0910 ±0·153	0·3120	12·09	28·59	2·59	9·02
			SODIUM CHLORIDE			
0·000	3·3390 ±0·109	0·3530	14·90	10·57	2·37	22·40
1·000	2·2430 ±0·150	0·3425	14·36	15·27	2·28	14·98
2·000	2·5100 ±0·107	0·3650	14·32	14·54	2·54	17·52
3·000	2·5390 ±0·081	0·3560	14·20	14·02	2·53	17·88
4·000	2·7650 ±0·084	0·3500	14·88	12·65	2·35	18·57
			MAGNESIUM CHLORIDE			
0·000	3·3390 ±0·109	0·3530	14·90	10·57	2·37	22·40
1·000	2·6040 ±0·241	0·5000	14·95	19·19	3·34	17·42
2·000	3·0790 ±0·181	0·3120	14·44	10·13	2·15	21·31
3·000	3·1700 ±0·152	0·2970	13·01	9·36	2·39	24·35
4·000	3·3470 ±0·116	0·2800	12·78	8·36	2·18	26·17
			CALCIUM CHLORIDE			
0·000	3·3390 ±0·109	0·3530	14·90	10·57	2·37	22·40
1·000	2·7560 ±0·184	0·4675	14·97	16·96	3·12	18·41
2·000	3·1410 ±0·115	0·3750	14·81	11·93	2·52	21·20
3·000	3·2280 ±0·086	0·3220	14·73	9·97	2·18	21·22
4·000	3·5960 ±0·172	0·2250	14·59	6·25	1·54	24·64

with rise in the concentrations of sodium and calcium chlorides due to their osmotic effect rather than any other specific action. Recently Gailey *et al.*[3] reported that 4 per cent sodium chloride solution stimulated spore formation in *Penicillium chrysogenum*. Since the osmotic pressure of the medium can be increased by increasing the concentrations of salts like sodium, potassium, calcium and magnesium chlorides, it was considered of interest to study the influence of high salt concentrations on fat formation by *P. aurantiobrunneum*.

P. aurantiobrunneum Dierckx was grown under static conditions on the following medium (g./100 ml.): sucrose, 30; NH_4NO_3, 0·45; $ZnSO_4.7H_2O$, 0·005; $FeCl_3.6H_2O$, 0·016; K_2SO_4, 0·044; $MgSO_4.7H_2O$, 0·5; $NaH_2PO_4.2H_2O$, 0·73; the requisite quantity of the salt; and distilled water. The *p*H of the medium was adjusted to 3·8. In these investigations a set of

TABLE 2 — EFFECT OF VARYING CONCENTRATIONS OF SODIUM AND CALCIUM CHLORIDES USED TOGETHER IN THE MEDIUM ON MYCELIAL WEIGHT AND FAT FORMATION

(Vol. of culture medium, 50 ml.; incubation period, 16 days; incubation temp., 25°C.)

Wt of $CaCl_2.6H_2O$ g./100 ml.	Wt of NaCl g./100 ml.	Wt of felt g.	Wt of fat g.	Wt of sugar consumed g.	Fat as % of felt	Fat coeff.	Economic coeff.
0·000	0·000	3·339 ±0·109	0·3530	14·90	10·57	2·37	22·40
0·500	0·500	2·500 ±0·099	0·4600	14·90	18·40	3·08	16·77
0·750	0·750	2·640 ±0·344	0·4700	14·82	17·80	3·17	17·81
1·000	1·000	2·784 ±0·185	0·8330	15·00	29·92	5·54	18·56
1·250	1·250	2·860 ±0·115	0·7620	15·00	26·64	5·08	19·06
1·500	1·500	3·571 ±0·083	0·5850	14·95	16·38	3·91	23·89

five 250 ml. flasks, each containing 50 ml. of the medium, was employed. These flasks were sterilized by autoclaving at 10 lb./sq. in. steam pressure for 15 min. The flasks were cooled, inoculated with mould spores and incubated at 25°C. for 16 days. The flasks were then autoclaved at 10 lb. steam pressure for 15 min., the felts removed, dried and fat extracted[4].

The effect of varying the concentration of different salts individually in the medium is indicated by the results recorded in Table 1. The results show that the weight of mycelial felt formed in the case of sodium, magnesium and calcium chlorides falls initially and then rises with increase in the concentration of the salts, whereas in the case of potassium chloride there is a gradual fall up to 2 per cent concentration of the salt and thereafter the fall is steep. Maximum fat formation is observed at 2 per cent concentration of sodium or potassium chloride, and is more in the case of potassium chloride. Maximum fat formation in the case of calcium and magnesium chlorides occurs at 1 per cent concentration of the salts, the amount of fat formed being more with magnesium chloride. In experiments employing sodium and calcium chlorides together in the medium (Table 2), the mycelial weight falls initially and then increases. Fat formation is highest (29·9 per cent) when the concentrations of the two salts are maintained at 1 per cent level. The amount of fat formed is more than that formed when the salts are used individually.

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Thyroid Status & Formation on Experimental Hydro-uteri in Rats

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Induced hypo- or hyperthyroidism has no effect on the formation of experimental hydro-uteri in rats. However, the concentration of some biochemical constituents of the accumulated uterine fluid is changed appreciably by such disturbance in thyroid status.

HYDRO-UTERI could be produced experimentally in different rodent species by ligating the uterus at uterotubal or cervical junctions or both, as well as by administration of estrogens[1,2]. The accumulated uterine fluid has been subjected to biochemical analyses[1-4]. The endocrine influence on the formation of hydro-uteri has also been demonstrated; ovariectomy has been found to retard but not prevent the development of this condition after uterine ligation[2].

The present report is concerned with the influence of thyroid status on the formation of experimental hydro-uteri in rats and on the biochemical composition of the accumulated fluid. For comparison, any parallel biochemical changes occurring in the serum have also been investigated.

Colony-bred albino rats of this Institute weighing 180-200 g. were used in this study. The left horn was ligated at the cervical junction under ether anaesthesia for production of hydro-uteri[2]. In hypothyroid animals the operation was performed 20 days after thyroidectomy. In hyperthyroid condition the uterus was ligated following 7 daily injections of 3,5,3′-triiodothyronine (12 μg./rat by

TABLE 1 — THYROID STATUS AND BIOCHEMICAL COMPOSITION OF THE RAT UTERINE FLUID

Treatment	No. of hydro-uterus and vol. of uterine fluid *ml.*	Composition of uterine fluid									
		Total cholesterol *mg./100 ml.*		Lactic acid *mg./100 ml.*		Glycogen *mg./100 ml.*		Nitrogen *mg./100 ml.*		Alkaline phosphatase *mg. P/ 100 ml./hr*	
		Uterine fluid	Serum	Uterine fluid	Serum	Uterine fluid	Serum	Uterine fluid	Serum	Uterine fluid	Serum
Controls (30)*	2 (0·6)†	242·0‡	434·0	3·75	37·5	82·0	220·0	528·0	1902·4	26·7	30·0
Thyroidectomized (30)*	2 (2·37)	160·0	527·0	3·0	38·8	82·5	330·0	592·0	2577·6	220·0	79·9
TIT (30)*	2 (0·5)	140·0	440·0	6·7	54·0	82·6	220·2	720·0	1955·2	276·17	26·6

*Number of animals.
†Total volume of uterine fluid from two rats.
‡Mean of two estimations based on pooled uterine fluid/serum from two rats.

the subcutaneous route); the hormonal regimen was continued for another 35 days from the date of operation. The animals were sacrificed on the 36th day after uterine ligation. The accumulated fluid was collected from the uterus for biochemical analysis by a sterilized tuberculin syringe. Parallel investigations on serum of such animals were carried out. Total cholesterol concentration was determined by some modifications of the method of Zlatkis *et al.*[5]. Lactic acid was estimated by the procedure of Barker and Summerson[6] and glycogen by the method of Kahan[7]. Nitrogen and alkaline phosphatase were measured by the procedures given by Hawk *et al.*[8].

It will be evident from the results presented in Table 1 that thyroid status had no effect on the formation of hydro-uteri; the incidence was the same (6·66 per cent) as in euthyroid animals. However, the total volume of the uterine fluid in the hypothyroid rats was somewhat higher than that in other groups. Under present experimental conditions, the concentration of cholesterol, lactic acid, glycogen and nitrogen in the uterine fluid was consistently lower than that in the serum. This was, however, not the case with alkaline phosphatase activity which was more or less similar in the two fluids, at least in euthyroid animals (Table 1). Any quantitative change in biochemical composition of the uterine fluid (due to disturbed thyroid status), independent of parallel fluctuations in the serum, was restricted to cholesterol, glycogen and alkaline phosphatase. Thus, hypo- or hyperthyroidism reduced cholesterol concentration in the uterine fluid in spite of the usual rise or no change in serum level. This reduction was more marked in hyperthyroid animals. Similarly, the glycogen concentration did not register any change in the uterine fluid but underwent a marked rise in the serum of the hypothyroid group. The situation was reversed with an increase in alkaline phosphatase activity in the uterine fluid of hyperthyroid animals and no appreciable change in the serum (Table 1). On the other hand, a rise in lactic acid and nitrogen concentration or an increase in alkaline phosphatase activity in the uterine fluid of hyperthyroid and/or hypothyroid animals were associated with changes of a similar nature in the serum. Such parallel alterations are compatible with the view that the uterine fluid is an ultrafiltrate of blood supplemented by endometrial secretions[1,2]. Whether any specific quantitative change in the concentration of a constituent of the uterine fluid (like cholesterol) is due to disturbed thyroid status *per se* or mediated via the pituitary-ovarian interplay, could not be hazarded from the present preliminary data.

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Reference to a thesis should include the name of author, title of the thesis (underlined), university or institution to which it was submitted and year of submission (e.g. CHANDRASEKHARAN, K. S., *Studies on Crystal Structure and Absolute Configuration of Crystals*, Ph.D. Thesis, Madras University, 1956).

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