Induction of a Cerebellar Disorder with Cycasin in Newborn Mice and Hamsters* (33933)

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(Introduced by G. L. Laqueur)
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While studying the carcinogenic effect of cycasin on newborn mice we noticed that many of the animals developed ataxia and hind-leg paralysis (1). Since the original report we have found that this effect is not limited to the original strain but is as readily obtained in a different strain of mice and in another rodent, namely the golden hamster. Moreover we can now describe the underlying pathologic process. The present paper summarizes the results of these studies.

Materials and Methods. Animals. Mice. Seventy-two pregnant mice of the C57BL/6 strain and 10 pregnant mice of the dd strain were used. The strains were originally obtained from the National Institute of Genetics, Mishima, Japan and bred in the laboratory. A total of 415 C57BL/6 and of 64 dd newborn mice resulted from these pregnancies. An additional 59 mice of the C57BL/6 strain were used when 2 months old to determine the effect of age on the development of the disorder. The newborn mice, together with their respective mothers, were arranged in groups as shown in Table I to determine the effect of age, dose, route of administration, and strain specificity. Mice were observed while alive for signs of neurologic abnormality. Histopathologic studies were performed on a separate group of 36 neonatal C57BL/6 mice which were killed in groups of 2 or 3 at intervals of 12–72 hr after a subcutaneous injection of cycasin (0.5 mg/g of body wt) within 24 hr after birth together with equal numbers of untreated controls of the same age.

Hamsters. Eighteen pregnant golden hamsters were obtained from the Central Laboratory of Experimental Animals, Tokyo, Japan and they gave birth to 150 newborns. The newborns and an additional group of 23 mature hamsters received cycasin at 3 dose levels and by two routes of administration as indicated in Table II.

Rats. Twenty-nine pregnant Moriyamaso rats gave birth to 276 rats all of which received a single dose of cycasin within 24 hr after birth at a level of 0.5 mg/g of body weight. Three additional pregnant rats received a single dose of 250 mg/kg of body weight on day 16 of gestation. Twenty-one young resulting from these pregnancies were observed for neurologic signs.

Chemical. Cycasin, methylazoxymethanol-β-D-glucoside, was obtained in crystalline form from the Faculty of Agriculture, Kagoshima University. The desired concentrations were prepared in physiologic saline and the solutions were filtered before use.

Diet. All animals except newborns and sucklings had free access to water and were maintained on a diet prepared by the Central Laboratory of Experimental Animals, Japan. Food was withheld from animals the night before they were given cycasin by stomach tube.

Histopathology. All animals with neurologic signs, whether sick or dead, were autopsied. The organs were fixed in 10% formalin and, following routine procedures of dehydration and embedding, the sections were stained with hematoxylin and eosin. Duplicate sections of the central nervous system were stained with Luxol fast blue, if indicated.

Results. Clinical observations. The grading of the severity of the neurologic signs was the same as previously described (1). Neurologic disorders were observed in mice and hamsters but not in rats. Moreover, animals were affected only when cycasin was administered within 24 hr after birth (Tables I and II). The severity of the disorders varied from

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TABLE I. Effect of Age, Dose, and Route of Administration of a Single Dose of Cycasin on Cerebellar Ataxia in C57BL/6 and dd Strains of Mice.

Expt. group	Strain of mice	No. and age of mice		Cycasin (mg/g of body wt)	Route of admin	No. of mice surviving 16 days	Severity of neurologic symptoms			
		Newborn	60 days				No sympt.	1	2	3
I	C57BL/6	270[a]		0.5	sc	171	17	31	65	58
II		47[b]		0.5	sc	34	34	0	0	0
III		33[c]		0.5	sc	29	29	0	0	0
IV		65		0.3	po	28	3	2	1	22
V			30[d]	0.3	po	26	26	0	0	0
VI			10[d]	0.5	po	5	5	0	0	0
VII			19[d]	1.0	po	6	6	0	0	0
VIII	dd	39		0.5	sc	37	37	0	0	0
IX		25		1.0	sc	13	4	2	4	3

[a] Included are 60 mice used in earlier report (1).
[b] Injected 48 hr after birth.
[c] Injected 96 hr after birth.
[d] The 3 groups of mature mice were included in earlier report (1).

slight weakness of the hind legs with excessive swaying of the hindquarters and slow gait (grade 1) to marked ataxia and a staggering gait during which the animals frequently stumbled or fell but were still able to rise (grade 3). The disorders were irreversible. There was no loss of bladder, anal or tail function in long-term survivors. Male and female mice were equally affected. Among mice receiving cycasin on the first postnatal day, neurologic signs occurred in about 90% of mice surviving 16 days regardless of the route of administration. In the dd strain, neurologic signs comparable to those observed in the C57BL/6 strain were noted only with a higher dose of cycasin (1 mg/g of body wt). The neurologic disturbance was more severe in hamsters than in mice. There

TABLE II. Effect of Age, Dose, and Route of Administration of a Single Dose of Cycasin on Cerebellar Ataxia in the Golden Hamster.

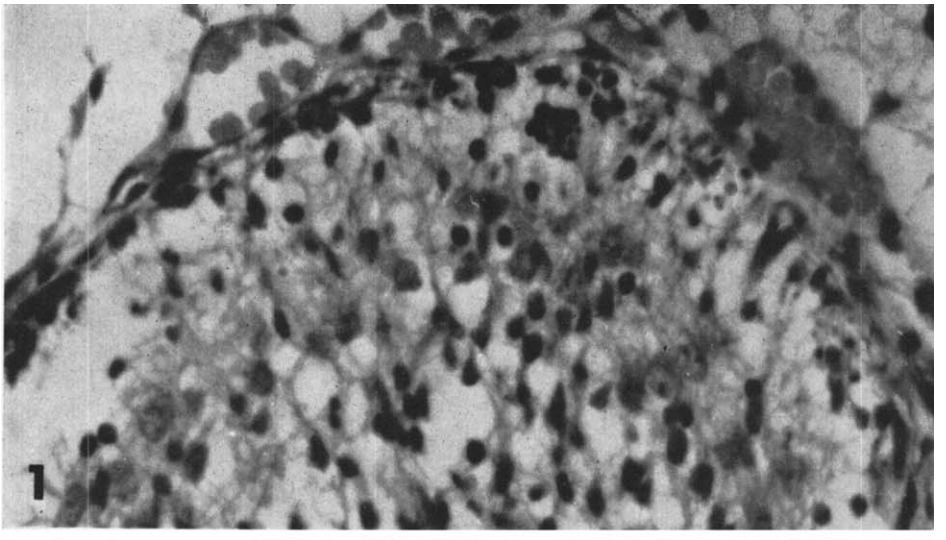
Expt. group	No. and age of hamsters		Cycasin (mg/g of body wt)	Route of admin	No. of hamsters surviving 16 days	Severity of neurologic symptoms			
	Newborn	Adult				No sympt.	1	2	3
X	96		0.2	sc	51	47	0	0	4
XI	40		0.4	sc	28	26	0	0	2
XII	14		0.6	sc	9	6	0	0	3
XIII		23	0.15	po	23	23	0	0	0

FIG. 1. Nuclear debris of the cells of the external granular layer, 60 hr after injection of cycasin into a mouse 24 hr after birth; hematoxylin–eosin; × 550.

FIG. 2. The cells of the external granular layer have nearly disappeared and only a few residual foci of necrosis are shown, 72 hr after injection of a newborn mouse; hematoxylin–eosin; × 550.

FIG. 3. The cells composing the external granular layer are greatly reduced in number, 72 hr after injection of a newborn (cf. Fig. 4); hematoxylin–eosin; × 70.

FIG. 4. Cerebellum of control mouse of same age as shown in Fig. 3; hematoxylin–eosin; ×70.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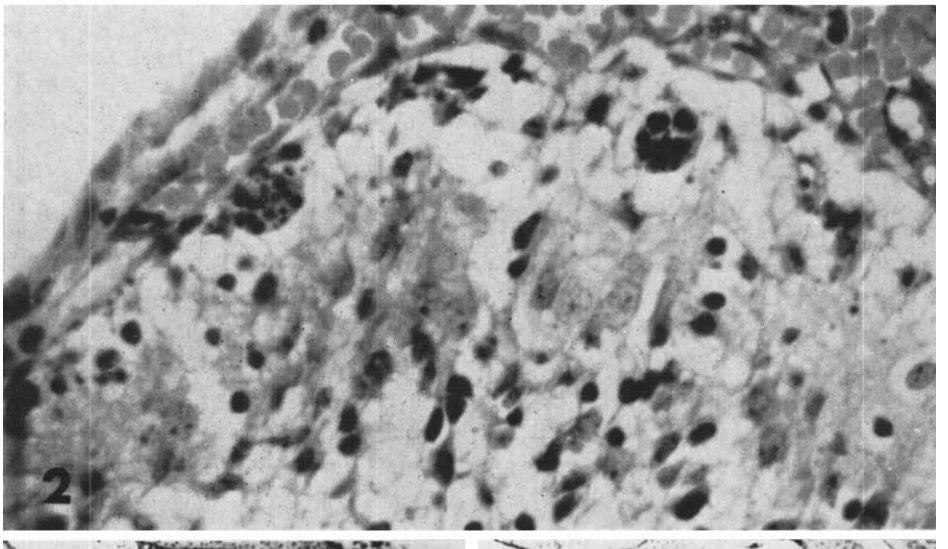
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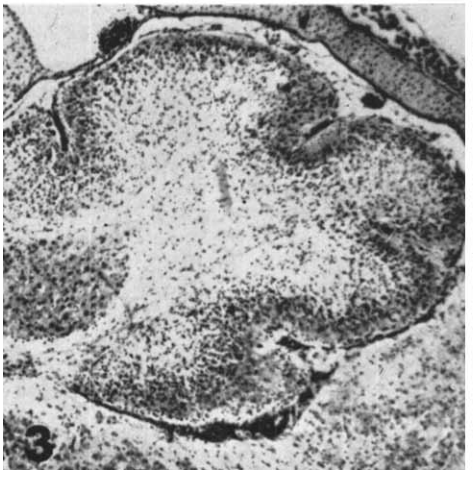
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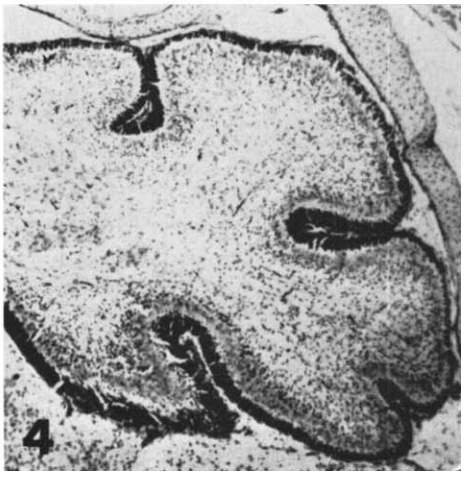
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was an additional observation that the majority of the affected hamsters did not squeal when caught whereas the unaffected hamsters usually did.

Anatomical observations. The pathologic changes in the brains of mice and hamsters receiving cycasin within 24 hr of birth were very similar for the two species, and they are described here only for mice. There was no significant difference on gross examination of the brain between experimentals and controls except for smoothness of the cerebellar surface in the mice with severe ataxia (grade 3).

Histopathologic findings were limited to the cerebellum. Maturation and postnatal organization of the cerebellum in control mice was complete at about the twentieth postnatal day as described by previous investigators (2, 3). In cycasin-treated animals, nuclear debris was found in the external granular layer 24 hr after the administration of cycasin. The necrotizing process progressed rapidly. Seventy-two hr after administration of cycasin the external granular layer had nearly disappeared (Figs. 1–4). Examination of the cerebellum between days 7 and 10 showed that the molecular and internal granule layers had failed to form, that Purkinje cells were scattered irregularly among the granule cells, and that there was a greatly reduced number of granule cells within the brain substance beneath the cortical surface. These changes persisted and were observed in mice as late as 260 days after the injection (Figs. 5–7).

Discussion. The irreversible neurologic disorder which was previously described in C57BL/6 mice after subcutaneous injection of cycasin into newborns (1) was not limited to this particular inbred strain. Identical effects were observed in the dd strain of mice and in a different species, the golden hamster. The similarity includes both clinical signs and histopathologic findings. Although the early mortality rates were high, a significant proportion of affected animals survived for months, thus making this prodedure suitable for long-term studies of cerebellar disorganization.

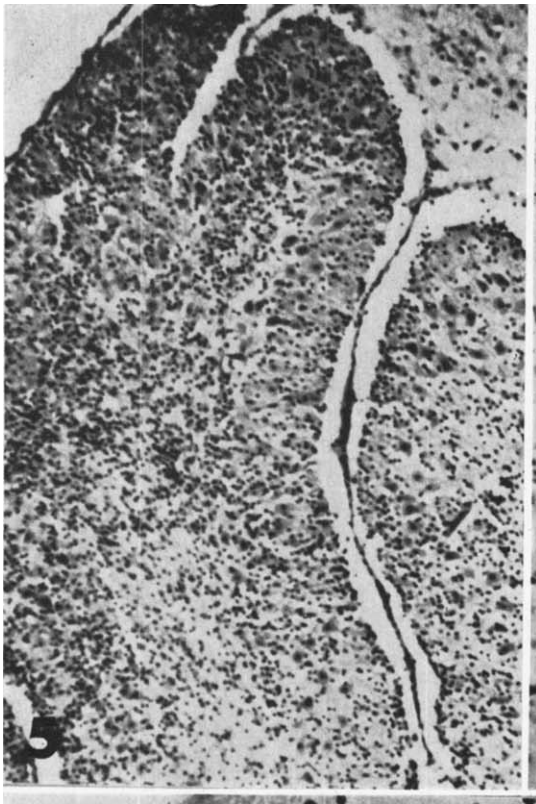
Successful induction of the disorder in mice and hamsters was limited to the first 24 hr of life. Enteric and parenteral routes were equally effective as shown in Table I (groups I and IV). In contrast to mice and hamsters, a group of identically treated rats of the Moriyamaso strain failed to develop the neurologic disease, even though the dose of cycasin was high enough to produce a mortality of 43%. The reason for failure to induce the neurologic disorder in rats during the first 24 hr of life is not known. The possibility exists that the timetable for maximal sensitivity of the cerebellar external granular layer in rats may differ from that of mice although exposure to X-irradiation in the first postnatal days was reported to induce similar cerebellar changes in both rats (4) and mice (5). Possibly a dosage schedule can be found that will produce cerebellar abnormalities in rats.

The primary site of injury leading subsequently to the neurologic disorder in mice was confined to the cells of the external granular layer, which underwent extensive necrosis. This effect occurred rapidly; necrotic cells were found in this layer within 24 hr. The ensuing disorganization of the cerebellar cortex might, therefore, be regarded as secondary to that injury. This interpretation is based on the observations of Uzman (2), Miale and Sidman (3) and Fujita *et al.* (6), who investigated the histogenesis of the mouse cerebellum and the patterns of cell migrations during the prenatal and postnatal periods. The main conclusions of these studies were that the external granular layer (situated on the surface of the cerebellum

FIG. 5. Scarcity of cells of the granule cell layer and displacement of Purkinje cells are the most prominent findings; the molecular layer is not distinguishable; 20 days after injection (cf. Fig. 6); hematoxylin–eosin; ×140.

FIG. 6. Cerebellar folium of control mouse of the same age as Fig. 5; development of the cerebellum has been completed; hematoxylin–eosin; ×140.

FIG. 7. Cerebellum of a mouse which survived 260 days after injection; this mouse had ataxia of grade 1; hematoxylin–eosin; ×550.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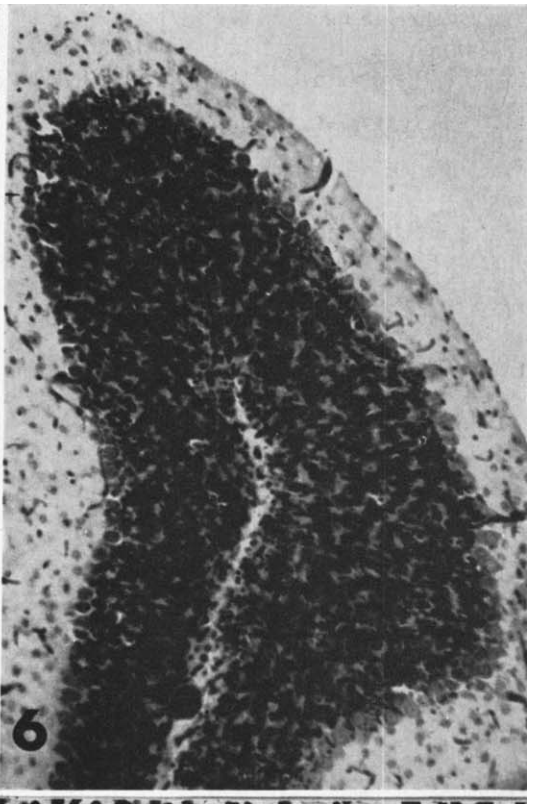
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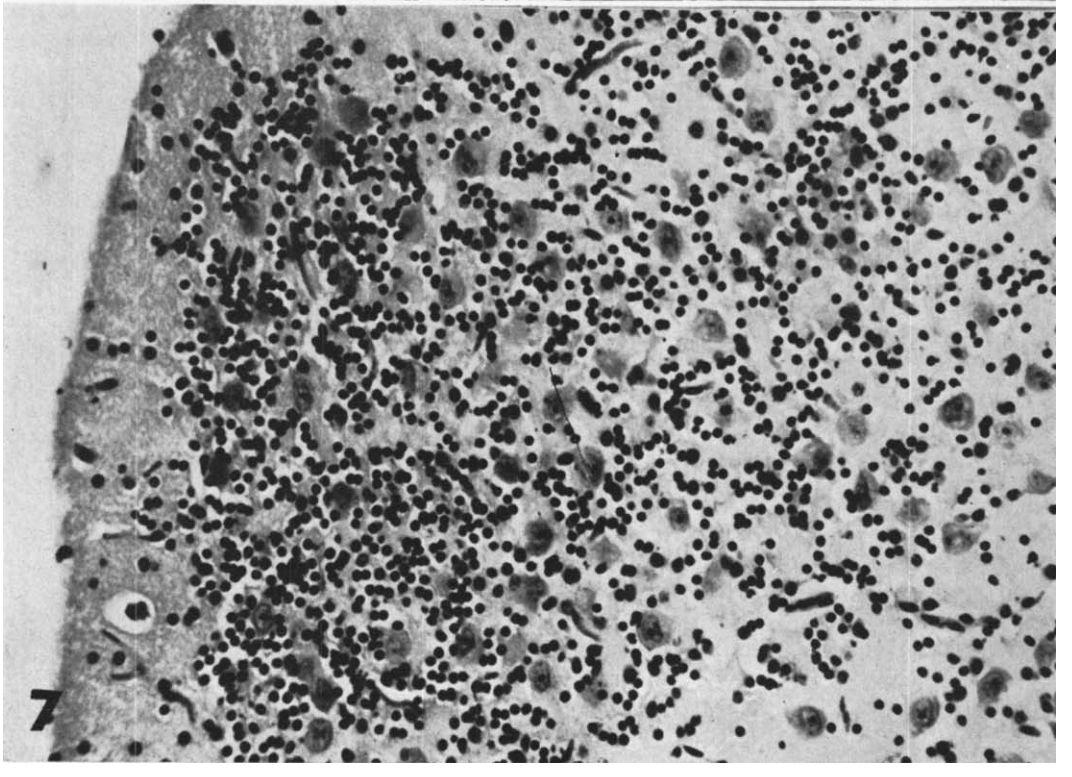
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beneath the pia mater at birth) provides the cells which form the molecular layer and most of the internal granule layer of the mature cerebellum, whereas Purkinje cells and roof nuclei originate in the subependymal matrix cells in the roof of the fourth ventricle. Thus the major cell types of the mature cerebellum arise from at least two separate groups of cells.

These conclusions are in good accord with the observations reported in the present study. We found that cycasin induced widespread necrosis of the external granular cells and that this resulted in an abortive formation of the molecular and granule cell layers of the cerebellum. Despite these profound changes there was basically no alteration in the development of those cellular components and nuclear aggregates which arise early in fetal development from the matrix of the roof of the fourth ventricle [see Table III of Ref. (3)]. It is notable that there is an extremely high level of cellular specificity in the toxic effects of cycasin on the cerebellum of the newborn animal. The striking selectivity of cycasin toxicity bears some resemblance to the selectivity of ionizing radiation and radiomimetic drugs.

The toxic effects of cycasin occur only after it is hydrolyzed to its aglycone, methylazoxymethanol (MAM). Upon oral administration of cycasin the hydrolysis is catalyzed primarily by β-glucosidase in intestinal bacteria (7). However, recent studies by Spatz (8) of very young rats indicate that this enzyme is present in the subcutaneous tissue during the late prenatal and early postnatal periods (8). Judging from the speed with which toxic effects were produced in mice and hamsters after subcutaneous administration of cycasin, it seems likely that a similar mechanism for cycasin hydrolysis exists in these animals.

The nature of the cytotoxic effect of cycasin remains to be determined in detail. It appears analogous, however, to the cytotoxic effect of MAM observed in liver cells (9) and in the cells of the subependymal matrix of the rat fetus (10). In the liver (12) and brain (Matsumoto, unpublished data) of rats treated with MAM there is methylation of the bases in RNA and DNA. The methylation of purified nucleic acids by MAM *in vitro* has also been reported (11). In liver cells of rats poisoned with MAM there are fine structural changes consistent with decreased synthesis of ribonucleic acids and protein (13).

Summary. A single administration of cycasin, β-D-glucosyloxyazoxymethane, was given in dosage near the LD_{50} level to newborn mice and hamsters within 24 hr after birth. Under these conditions cycasin produced a distinctive neurologic disorder. In the affected newborns there was rapid and extensive necrosis of the cells of the external granular layer (embryonal layer) of the cerebellum. In animals surviving to maturity this resulted in defective development of the molecular and granule cell layers. The affected animals had ataxia and gait disturbances. Comparable administration of cycasin to rats within 24 hr after birth produced no apparent disorder of the central nervous system.

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The Extinction Coefficient of Canine Fibrin in Alkaline Urea at 282 mμ* (33934)

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The measurement of the concentration of fibrinogen in plasma by an isotope dilution method (1) requires the determination of the specific radioactivity (counts per minute per milligram of protein) of fibrin in alkaline urea. The concentration of fibrin (mg/ml) is obtained from the measurement of the optical density of an alkaline urea solution of this protein and the use of an extinction coefficient. This extinction coefficient has been measured for fibrins in man and the cow (2) and in rabbit (1) and the results show close agreement among these species.

In a study of the metabolism and distribution of fibrinogen in beagle dogs (3) all fibrinogen concentrations were determined by the isotope dilution method. Since no data exist, to the knowledge of the authors, on the extinction coefficient of canine fibrin dissolved in alkaline urea, it was considered desirable to determine this value rather than rely on a value obtained from other species.

Methods and Results. Fibrinogen from the plasma of 4 mongrel dogs was isolated, purified, and the coagulability was determined according to the method of Atencio *et al.* (1). A thrombin (Parke, Davis & Co., Detroit, Mich.) solution containing 8–10 NIH units of thrombin was added to the fibrinogen solution and the fibrin was collected on a siliconized glass rod by carefully winding the rod against the side of the glass tube. The fibrin on the glass rod was then dialyzed free of salts by repeated changes of distilled water and was then transferred from the glass rod into the bottom of a tared volumetric flask. The fibrin was then dehydrated by the addition of 10-ml volumes of a mixture of ethanol and ether (v/v, 3 parts to 2 parts) 5 times. The flasks containing the fibrin were dried to constant weight at 110°. Freshly prepared alkaline urea (40% urea in 0.2 *N* NaOH) was added to each flask to dissolve the fibrin, and the optical density of samples containing different amounts of fibrin was measured in 1-cm quartz cuvettes at 282 mμ in a Beckman DU spectrophotometer (Beckman Instrument Co., Fullerton, Calif.).

The mean percentage coagulability of the purified fibrinogen from the 4 dogs was 95.3%.

A plot of the 30 measured optical densities against the concentration of fibrin in alkaline urea is shown in Fig. 1. Least square analysis reveals that the equation $y = 1.743x + 0.015$ (solid line in Fig. 1) best describes the data (dots) and the correlation coefficient between optical density and fibrin concentration is 1.0 (0.9998). The standard deviation of a single value from the calculated curve is ±0.009.

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