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Mammary Gland Growth in the Hypophysectomized Pregnant Rat[1, 2] (38522)

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Growth of the mammary gland during the first 20 days of pregnancy in the rat may be mimicked by injecting 2 μg estradiol benzoate and 6 mg progesterone subcutaneously into ovariectomized rats each day for 19 days (1). This observation led to the concept that mammary gland growth is dependent primarily upon the ovarian steroid hormones in synergism with a mammotropic hormone of anterior pituitary origin. However, the involvement of the placenta in hypophysectomized rats and mice as a stimulator of mammary gland growth has been demonstrated (2–4). Attempts to extract and concentrate placental substances with mammotropic and/or luteotropic properties have been partially successful (5–8) or unsuccessful (9).

Objectives of the present study were to determine the amount of mammary gland growth that would occur when the pituitary gland was removed at mid-pregnancy in the rat and to determine whether or not a relationship between the amount of placental tissue present and the quantitative growth of the mammary gland was demonstrable. Chemical determination of deoxyribonucleic acid (DNA) served as the index for mammary gland growth.

Materials and Methods. Mature female albino rats of the Sprague–Dawley–Rolfsmeyer strain were maintained in colony cages of five animals per cage at a constant temperature of 25.6 ± 1° with 14 hr of artificial light each day and free access to Purina Lab Chow and tap water. Vaginal smears were obtained each morning by vaginal lavage and at least one full estrous cycle was followed prior to mating. The day in which sperm were detected in the smear was designated day 1 of pregnancy.

In the first experiment, animals were hypophysectomized on days 11, 12 or 15 of pregnancy by the parapharyngeal approach to the pituitary gland. Completeness of hypophysectomy was assessed with the aid of a dissecting microscope at the time of operation and again upon autopsy on day 20 when the animal was sacrificed. The six abdominal–inguinal mammary glands were removed and frozen immediately for later analysis of DNA (10) and ribonucleic acid (RNA) by methods described previously (11).

In the second experiment, rats were hypophysectomized on day 12 of pregnancy and fetuses and placentas removed immediately afterwards via a ventral midline abdominal incision and small uterine incisions. One fetus and one placenta (placental–fetal unit) remained in each of six rats, two placental–fetal units in another six rats, and so on, to five placental–fetal units per rat. The fetuses and placentas were removed starting from the tubouterine end toward the cervical end so that the units remaining were closer to the cervical end of the uterine horn. The animals were sacrificed on day 20 of pregnancy and mammary glands removed for subsequent nucleic acid determinations.

A third experiment was designed to determine the extent of mammary gland growth in rats in which the pituitary was retained, while having the numbers of placental–fetal units varied. Groups of five rats each had placental–fetal units removed on day 12 of pregnancy so that those remaining numbered 0, 1, 2, 3, 4 or 5 in each of 6 groups. The animals were sacrificed on day 20 of pregnancy and mammary glands were obtained.

In a fourth experiment, rats were hypophysectomized on day 12 of pregnancy and were also laparotomized to remove all fetuses and all except 0, 1, 2, 3, 4 or 5 placentas.

The results of the fourth experiment led to the fifth and final experiment in which

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TABLE 1. NUCLEIC ACIDS OF SIX ABDOMINAL–INGUINAL MAMMARY GLANDS FROM HYPOPHYSECTOMIZED-PREGNANT RATS.

Group	Day of preg sacrificed	No. of rats	Final body wt (g)	No. of fetuses	Wt wet of m.g.[b] (g)	DFFT wt of m.g.[b] (mg)	DNA/ 100 g bw (mg)	RNA/ 100 g bw (mg)
Normal preg	12	12	261	8.1	5.1	492	4.70	3.17
Normal preg	20	12	286	7.9	8.2	680	7.30	6.30
Sham operated								
D-12	20	10	273	7.8	7.9	585	6.14	5.94
Hypophysectomized								
D-11	20	11	266	7.3	6.4	565	6.10	5.49
D-12	20	10	277	7.7	7.3	6.12	6.62	6.27
D-15	20	11	281	8.0	7.7	648	6.86	5.51
Virgin control	—	12	240	—	3.1	337	3.00	2.20
Aborted D-12, 13 or 14 (8 rats hypoxed D-11; 3 rats hypoxed D-12)	20	11	255	—	3.3	351	3.05	1.89
LSD[a]							1.08	1.19

[a] Group means tested by Least Significant Difference (LSD) ($P < 0.05$) (12).
[b] m.g. = Mammary gland.

five rats per group were hypophysectomized on day 12 of pregnancy but were not laparotomized until day 14 when all fetuses and all except 0, 1, 2, 3, 4 or 5 placentas were removed in respective groups. In this experiment animals were sacrificed on day 20 of pregnancy and mammary glands taken.

Group means in experiments 1 through 5 were analyzed statistically by analysis of variance and the least significant difference (LSD) test (12).

Results. The DNA content of abdominal–inguinal mammary glands of normal pregnant rats sacrificed on day 20 of pregnancy was 7.30 ± 0.54 mg/100 g bw (Table I). Animals that were sham-operated on day 12 or hypophysectomized on day 11 of pregnancy had mammary gland DNA contents that were significantly lower than the intact pregnant control group. The RNA content of the mammary gland during pregnancy was slightly lower than the DNA content. Although the RNA contents of the sham-operated and hypophysectomized groups were lower than the intact pregnant control, the differences were not significant. The virgin control groups had significantly lower nucleic acid contents in the mammary glands than any of the groups which maintained pregnancy to day 20. Eight of the rats hypophysectomized on day 11 of pregnancy and three of the rats hypophysectomized on day 12 aborted within several days after the operation. These animals were retained to the day which would have corresponded to day 20 of pregnancy had they not aborted. The nucleic acid contents of their mammary glands were found to be similar to the virgin control animals.

The results of DNA determinations of the mammary glands on day 20 of pregnancy following removal of all but one to five placental–fetal units on day 12 are presented in Fig. 1. It may be seen that the DNA content of six mammary glands of animals carrying only one fetus and placenta from day 12 to day 20 was 4.83 ± 0.24 mg/100 g bw, which compared to 4.70 ± 0.29 for day 12 of normal pregnancy. When two placental–fetal units were retained in the uterus to day 20 the DNA was 5.66 ± 0.21 mg/100 g bw, nonsignificantly higher than day 12 normal pregnancy and nonsignificantly lower than day 20 of pregnancy after hypophysectomy on day 12. Three, four or five placental–fetal units resulted in mammary gland DNA contents which were not significantly different from the day 20 pregnant group

that was hypophysectomized on day 12, but these groups were significantly higher ($P < .05$) than the group having only one conceptus from day 12 to day 20.

In the third experiment the pituitary glands were kept intact while the numbers of placental–fetal units on day 12 of pregnancy were varied so that from zero to five remained until day 20 when the animals were sacrificed. Under these circumstances the mammary gland DNA was not different from the normal control group in any group having from one to five placental–fetal units (Table II).

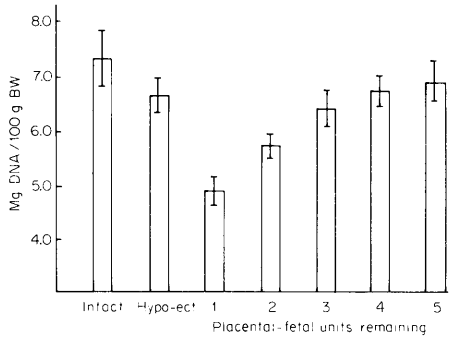

FIG. 1. Mammary gland DNA (mg/100 g body weight) in 6 abdominal-inguinal glands of rats euthanized on day 20 pregnancy after hypophysectomy (Hypo-ect) plus all but the indicated numbers of placental–fetal units removed on day 12 pregnancy.

The fourth experiment was designed to eliminate any role played by the fetus on mammary gland growth. The pituitaries were removed on day 12 of pregnancy and the animals were laparotomized to remove all fetuses and all except one to five placentas. In every case, the animal died within 60 hr after the operation. Death was attributed to excessive hemorrhaging, but other unknown factors may have been significant, such as a placental toxicity that cannot be tolerated by the hypophysectomized animal.

An attempt to overcome the failure of the fourth experiment was made in the fifth experiment. Rats were hypophysectomized on day 12 of pregnancy but fetuses and placentas were not removed until day 14. On this day the animals were laparotomized and all except zero to five placentas were retained. The results indicated that a significantly lower mammary gland DNA was evident when one placenta remained compared to two to five or more (controls) placentas remaining *in utero* to day 20 (Table III).

Discussion. Selye *et al.* (3) demonstrated that mammary gland maintenance occurred in the absence of the pituitary during the latter half of pregnancy in the rat. The observations were morphological and not readily amenable to quantitative evaluation.

TABLE II. MAMMARY GLAND CHANGES OF RATS HAVING INTACT PITUITARIES WITH NUMBERS OF CONCEPTUSES *IN UTERO* FROM DAY 12 TO DAY 20 OF PREGNANCY ALTERED WITH 0–5 REMAINING.

Groups[a]	b.w.[c] (g)	m.g.[d] wet wt (g)	Placental disc wet wt (mg)	m.g. DFFT (mg)	Total DNA (mg)	DNA/100 g b.w. (mg)
(1) 0 conceptus	229	2.94	—	251	8.45	3.65
(2) 1 conceptus	271	7.22	405	571	19.99	7.31
(3) 2 conceptus	284	9.06	776	857	22.01	7.83
(4) 3 conceptus	280	8.82	995	641	19.63	7.00
(5) 4 conceptus	266	6.66	1175	498	20.58	7.75
(6) 5 conceptus	277	7.40	1125	543	22.17	7.81
(7) Normal preg Cont. Day 20	275	7.84	1334	622	20.89	7.54
(8) Normal virgin Controls	224	3.06	—	305	7.56	3.35
LSD[b]	18	1.30	226	160	3.78	1.24

[a] Five rats per group.
[b] Groups means tested by Least Significance Difference (LSD) ($P < .05$) (12).
[c] b.w. = Body weight.
[d] m.g. = Mammary gland.

TABLE III. MAMMARY GLAND CHANGES OF RATS WITH PITUITARY REMOVED ON DAY 12 AND WITH NUMBERS OF PLACENTAS *IN UTERO* FROM DAY 14 TO DAY 20 OF PREGNANCY ALTERED WITH 0–5 REMAINING.

Groups[a]	b.w.[c] (g)	m.g.[d] wet wt (g)	Placental disc wet wt (mg)	m.g. DFFT (mg)	Total DNA (mg)	DNA/100 g b.w. (mg)
(1) 0 placentas	240	3.46	—	312	9.12	3.76
(2) 1 placenta	258	6.26	302	481	17.52	6.73
(3) 2 placentas	261	5.92	755	474	20.45	7.90
(4) 3 placentas	253	6.54	811	464	20.55	8.11
(5) 4 placentas	261	6.16	1098	458	20.71	8.02
(6) 5 placentas	281	7.04	1207	556	22.80	7.95
(7) Normal preg cont. Day 20	263	6.38	1374	587	20.41	7.74
(8) Normal virgin controls	231	2.98	—	289	7.25	3.12
LSD[b]	19	1.21	316	108	2.32	0.93

[a] Five rats per group.
[b] Group means tested by Least Significant Difference (LSD) ($P < .05$) (12).
[c] b.w. = Body weight.
[d] m.g. = Mammary gland.

However, the evidence was convincing in implicating the placenta as a source of mammotropic hormone. Later, Weichert and Schurgast (13) measured the size of corpora lutea during various stages of pregnancy in the rat and demonstrated a decided stimulus to growth of the corpora lutea (luteotropic hormone) at day 12 of pregnancy. It was speculated that this stimulus originated from the placentas of the rats. More recent investigations by Cohen and Gala (14) have reinforced the earlier work by demonstrating the presence of luteotropic and mammotropic activities in the serum of rats on day 12 of pregnancy. Since total ovariectomy cannot be performed during pregnancy in the rat without abortion resulting, it would appear that the placental luteotropin is responsible for the added stimulus which is evidenced on day 12 of pregnancy and that this luteotropin of placental origin is a sufficient stimulus to maintain steroidogenesis in the corpora lutea for the remainder of pregnancy.

The mammotropic activity of rat placentas taken at day 12 of pregnancy has been demonstrated in maternal blood on day 12 of pregnancy (14, 15) and the trophoblast of day 12 placentas (15). The assays were based on whole-mount observations of lobule-alveoli of the mammary gland and as such were not as objectively quantitative as the DNA determinations used in the present study. However, it was observed by Matthies (15) that a readily assayable amount of mammotropic activity was present in a single day-12 rat placenta. The present study supports his observation and indicates that a minimum of three placental–fetal units is required to produce sufficient luteotropic and mammotropic activities to support mammary gland growth during the latter half of pregnancy not significantly different from normal.

Although George *et al.* (16) reported a lack of mammary growth in late pregnancy after hypophysectomy on day 14, they did not have suitable controls, such as a day 14 pregnant group or a group sham-operated on day 14 and sacrificed on day 20, to verify their conclusions. Based on the data presented by them, an alternative conclusion would be that the stress of hypophysectomy only delayed maximal mammary development for a few days. This conclusion is borne-out by my data presented in Table I.

My data may be interpreted to mean that only a single placental–fetal unit produces sufficient luteotropin to maintain the corpora lutea at a level which will prevent abortion,

when the pituitary is absent but at least three placental–fetal units are needed in the critical period between days 12 and 14 of pregannncy to provide the added stimulus to the corpora lutea for sufficient steroidogenesis, as well as to provide a level of mammotropin necessary to synergize with the steroids for normal mammary gland proliferation.

Summary. Sprague–Dawley–Rolfsmeyer rats were hypophysectomized on days 11, 12 or 15 of pregnancy and sacrificed on day 20 to determine the extent of mammary development, as assessed by determination of nucleic acid content. The DNA of six abdominal–inguinal glands in the hypophysectomized groups was not significantly different from that in the sham-operated pregnant or intact pregnant control groups. All groups maintaining pregnancy had significantly higher DNA contents in mammary glands than virgin control or hypophysectomized-aborted groups. In order to determine the minimal numbers of placental–fetal units required to maintain pregnancy and mammary gland growth, fetuses and placentas were removed on day 12 of pregnancy in addition to the pituitary so that only one fetus and one placenta remained in the uterus of a group of 6 rats with other groups having 2, 3, 4 or 5 remaining. Pregnancy was maintained with only one placental–fetal unit, but mammary gland proliferation was significantly lower than the control group on day 20 of pregnancy. Three to five conceptuses supported mammary proliferation during the latter half of pregnancy at a level not significantly different from intact or sham-operated control groups. Removal of placental units on day 12 in rats having pituitaries intact resulted in no mammary DNA change when 1–5 units remained. Removal of pituitaries on day 12 and placental-fetal units on day 14 also resulted in no change in mammary DNA with as little as two placentas (minus all fetuses), while only one placenta remaining resulted in a significantly lower mammary DNA than in groups with 2 or more placentas.

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Serum Hexosaminidase Activity in Man Under Simulated Diving Conditions[1] (38523)

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Several enzyme activities have been studied in the sera of animals and humans exposed to a variety of hyperbaric conditions (1–4). The metabolic defect in a number of sphingolipid storage diseases known today is an attenuation or complete absence of a specific hydrolytic enzyme required for the catabolism of a lipid (5). Our previous results showed that there were changes in the amounts of sphingoglycolipid in the blood and tissues of rats following hyperbaric exposure (6). Therefore, an investigation of enzyme activity associated with sphingoglycolipid metabolism in the serum of human divers was initiated. Hexosaminidases were measured, since there is a considerable amount of hexosaminidase A and B activity in serum which can be quantitatively measured with artificial substrates (7).

Materials and Methods. All dives were conducted in the hyperbaric chamber complex of the Naval Experimental Diving Unit, Washington, D.C. The following dives were performed:

Saturation dive at 1000 feet of sea water (FSW). Four subjects were compressed at a rate of 5 ft/min on a mixture of helium and oxygen to an ultimate saturation depth of 1000 FSW. During compression, three day intermediate stops were made at 200, 400, 600 and 800 FSW. The divers remained four days at a saturation depth of 1000 FSW. Decompression was performed in accordance with the standard U.S. Navy format (Table I), with the exception of a 24 hr stop at 850 FSW to permit physiological studies.

Subsaturation dives at 650 FSW. Fourteen divers, in three successive dives, were compressed on a helium and oxygen mixture to a simulated depth of 650 FSW at a rate of 30 ft/min to 400 FSW, 15 ft/min from 400 to 600 FSW, and 10 ft/min from 600 to 650 FSW. Following a 3.5 hr holding period at this depth, rapid decompression was performed. In all three dives, however, the initial rapid decompression resulted in decompression sickness at a depth of 430–470 FSW in one or more subjects, necessitating therapeutic recompression to 650 FSW and overnight saturation at that depth. Subsequent decompression from 650 FSW was performed in accordance with the schedule in Table I.

Subsaturation dive at 400 FSW. Eight divers were exposed to a mixture of helium–oxygen at a simulated depth of 400 FSW on two successive dives. After a 3.5 hr holding period the chamber was decompressed on an experimental schedule.

During these helium–oxygen dives, the chamber atmosphere was monitored continuously for oxygen and carbon dioxide content, temperature and relative humidity. Oxygen concentration was maintained between 0.29 and 0.35 atmosphere, carbon dioxide content was not allowed to exceed 0.5 % surface equivalent, temperature ranged from 26° to 32°, and relative humidity from 50 to 70 %.

Fasting blood samples were obtained by venipuncture at 7 AM on the days indicated (Figs. 1–3). The blood samples that were drawn at increased ambient pressure were decompressed at a rate of 15 ft/min. Prior to centrifugation, samples were stored in an ice bath. After clotting, samples were centrifuged at 500 *g* for 5 min at room temperature and the serum was separated.

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TABLE I. RATE OF DECOMPRESSION FROM SATURATION EXPOSURES ON HELIUM–OXYGEN.

Depth (ft sea water)	Rate[a] (ft/hr)
Initial 30 ft ascent	10
1000–200	6
200–100	5
100–50	4
50–Surface	3

[a] Decompression is interrupted daily between 1400 and 1600 hr and between 0000 and 0600 hr.

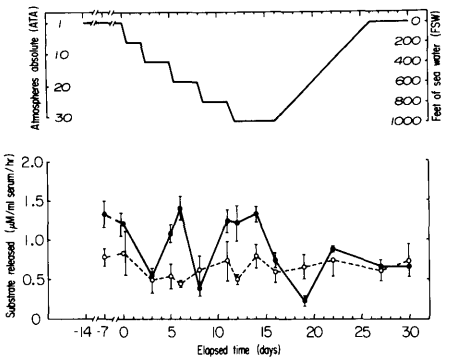

FIG. 1. Serum hexosaminidase levels during a 1000 FSW saturation dive. Upper section: diving schedule; lower section: enzyme activity, hexosaminidase A (●); hexosaminidase B (○), values are the mean ± standard error.

Hexosaminidase A and B activities were measured by the procedure of O'Brien (7). Total hexosaminidase activity was measured using 10 μl of serum diluted with 50 μl of 0.1 *M* citrate–phosphate buffer (pH 4.4). One hundred μl of 10 m*M* 4-methylumbelliferyl-*N*-acetyl-β-glucosamine (Sigma Chemical Co., St. Louis, MO) dissolved in the buffer described above was added to each sample. After incubation at 37° for 30 min, 5 ml of 0.17 *M* glycine–NaOH buffer (pH 10) was added to stop the reaction. Fluorescence was determined in an Aminco–Bowman spectrophotofluorometer at an excitation wavelength of 366 nm and an emission wavelength of 446 nm. To measure hexosaminidase B activity, the same conditions were used as described above except the serum was preincubated for 4 hr at 50° to inactivate hexosaminidase A selectively. A standard curve was determined using 4-methyl-umbelliferone dissolved in 0.17 *M* glycine–NaOH buffer over a range of 0–10 nmoles. The extent of substrate cleavage was determined by comparison of the fluorescence of the samples against the standards. All samples were measured in duplicate. The activity of hexosaminidase A was calculated as the difference in total activity and that activity remaining after heating at 50° for 4 hr.

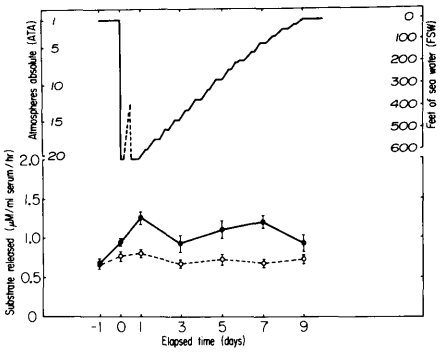

FIG. 2. Serum hexosaminidase levels during 650 FSW subsaturation dives. Upper section: diving schedule; lower section: enzyme activity, hexosaminidase A (●); hexosaminidase B (○), values are the mean ± standard error.

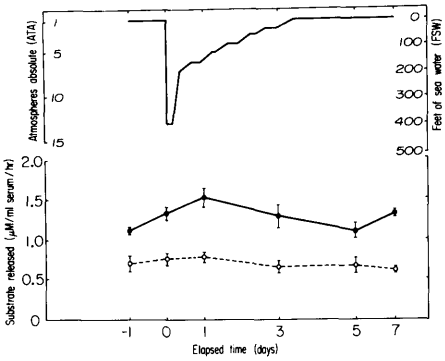

FIG. 3. Serum hexosaminidase levels during a 400 FSW subsaturation dive. Upper section: diving schedule; lower section: enzyme activity, hexosaminidase A (●); hexosaminidase B (○), values are the mean ± standard error.

Results. One thousand FSW dive. The activities of hexosaminidases A and B in the sera of divers in a 1000 FSW saturation dive are shown in Fig. 1. All four divers had very similar patterns of change. The mean activity of hexosaminidase A decreased more than 50% at the end of compression from 200 FSW to 400 FSW and returned gradually to normal, then dropped again at 600 FSW.

The enzyme activity remained in the normal range at 800 FSW and in the first part of the 4-day saturation period at 1000 FSW. A further decrease in hexosaminidase A activities was observed at the end of the 4-day saturation period at 1000 FSW and in the early stage of decompression. There were no remarkable changes in the mean values of hexosaminidase B.

Six hundred-fifty FSW dive. Figure 2 shows the changes in the mean value of serum hexosaminidase activities during the 4-hr subsaturation dives at 650 FSW. Ten of the divers reported compression arthralgia and all divers reported symptoms of the high pressure nervous syndrome, including tremors and unsteadiness. During the initial decompression at 650 FSW, four of the fourteen divers developed severe decompression sickness requiring therapeutic recompression at 650 FSW and overnight saturation. There were increases in mean values of hexosaminidase A activities at the depth of 650 FSW after recompression and at the depth of 300 FSW and 100 FSW during decompression, whereas no changes were found in the mean values of hexosaminidase B activities.

Four hundred FSW dive. Figure 3 illustrates the hexosaminidase activity for the 400 FSW dive. A slight increase in hexosaminidase A activities was observed at a depth of 150 FSW during decompression. Hexosaminidase B activities fluctuated within the normal range.

Discussion. Earlier studies showed that hexosaminidases are enzymes responsible for catabolism of ganglioside GM_2 and globosides and can be used as a marker in sphingolipid storage diseases (5). Changes in glycolipid metabolizing enzymes associated with hyperbaric exposure have not been investigated to our knowledge. In this study, an increase in hexosaminidase A activity was observed 24 hr after the compression–decompression cycle in the 400 FSW and 650 FSW dives. In contrast, in the 1000 FSW dive a marked decrease in hexosaminidase A activity was observed at 400, 600 and 1000 FSW during compression and during the early stages of decompression. The functional changes that may occur in cells or tissues related to the observed changes in serum enzyme activities at extreme depths cannot be explained at this time. We cannot account for the widely differing profiles of our observations in the three dives. It appeared that the changes might be related to the single or combined effects of increased ambient pressure, compression or to the effects of decompression. Also, individual responses of the subjects can vary greatly. Uddin *et al.* (3) have reported that changes in serum creatine phosphokinase (CPK) activity were observed during hyperbaric exposure and suggested that both compression and decompression stress, if sufficiently severe, may produce elevations in CPK activity.

The procedures for measuring glycolipid metabolizing enzymes are sensitive enough to permit detection of minor changes of serum enzyme activity under high stress conditions. However, the site of action, amplification of the observed responses and the functional changes related to the enzyme activity, and lipid changes remains to be determined.

Summary. The activities of serum hexosaminidases from human divers before, during and after simulated dives was measured. Decreases in hexosaminidase A activities were observed in the 1000 FSW saturation dive, whereas an increase in hexosaminidase A activity was observed during decompression in the subsaturation dives at 400 FSW and 650 FSW.

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The opinions and statements contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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Photodynamic Inactivation of Herpesvirus Hominis by Methylene Blue (38524)

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Genital and other infections caused by herpesvirus hominis have become increasingly common during recent years. The incidence of genital involvement by this agent among 12,647 cases seen in the Skin Clinic of the Tufts-New England Medical Center during 1972–73 was 0.8% (1). Therapy has presented considerable difficulty not only in eradicating primary mucocutaneous infection but also in decreasing their occurrence to an appreciable degree. Without simultaneous application of dimethyl sulfoxide, topical treatment with idoxuridine application has produced poor clinical results (2–4). A controlled study of the effect of locally applied neutral red plus light in patients with superficial herpetic lesions has indicated that the clinical course is shortened and the frequency of relapse reduced (5). Proflavine, a derivative of acridine, has produced similar results; however, a controlled study of the use of this agent has not been carried out. Methylene blue, a compound structurally resembling acridine, has been shown to produce photodynamic killing of a number of bacterial genera and species (6). It is not known, however, whether this dye acts on viruses in a similar manner.

The purposes of the present investigation were to (a) examine the photodynamic activity of methylene blue against herpesvirus hominis, (b) determine the sensitivity of clinical isolates of the virus to photoinactivation, and (c) study the development of resistance to photoinactivation by dyes and light. Because methylene blue, proflavine, and neutral red are all tricyclic compounds, the effects of these were also investigated.

Materials and Methods. In vitro sensitivity to photoinactivation. Thirty-seven fresh isolates of herpesvirus hominis, 29 recovered from genital and eight from oral lesions, were examined for sensitivity to proflavine, neutral red and methylene blue in the presence of light by the method described by Wallis and Melnick (7). The dye was dissolved in triple distilled water to make a 10^{-3} *M* solution, and sterilized by boiling for 15 min. Boric acid buffer (pH 9.0) was added in a volume to yield a dye concentration of 10^{-5} *M*. Dowex cation exchange resin, 50W-X4, 50–100 mesh hydrogen form was repeatedly washed with 0.85% NaCl until a pH of 6–6.5 was reached. It was then packed into 13 × 100 mm tubes to a height of 35 mm, sterilized by boiling for 15 min and washed repeatedly with sterile boric acid buffer at pH9 until pH of the supernatant was constant at 9.0. The fluid was then removed from the tubes.

The photosensitizing procedure was carried out in a room lighted by red lights. 4.5 ml of 10^{-5} *M* dye solution (pH9) was placed in a tube shielded by aluminum foil. One-half milliliter of virus was then added and the mixture incubated at 37° for 1 hr. A virus control was treated in similar fashion.

One-half of the supernatant (virus-dye complex) was stored in the dark, while the other half was exposed to a daylight fluorescent lamp (2 15-W tubes by Westinghouse) at a distance of 15 cm for 5 min.

The virus from both the exposed and non-exposed tubes was then titrated in primary human amnion cell cultures maintained in Eagle's minimal essential medium containing 2% fetal calf serum. All the cultures were covered by aluminum foil and incubated for 7 days before reading.

The development of resistance to photoinactivation. Twenty strains of the virus were tested for the development of resistance *in vitro* by repeated passage in tissue culture in the presence of neutral red, methylene blue or both. Dye–virus mixtures were studied in duplicate. One set was kept in the dark, while the other was exposed to 15-W fluorescent lamp at a distance of 15 cm for 10 min.

TABLE 1. *In Vitro* SENSITIVITY OF HERPESVIRUS TO METHYLENE BLUE, NEUTRAL RED AND PROFLAVINE.

Photosensitivity	Virus titer			Methylene blue	Neurtal red	Proflavine
	Control	dark	light			
High	6–8[a]	6–8	0	27[b]	26	2
Moderate	6–8	6–8	3–5	4	5	4
Low	6–8	6–8	5–7	6	6	2
Activity in dark	6–8	0–5	0–3	0	0	14
Total strains				37	37	22

[a] Range of TCD-50, No. of negative log.
[b] Number of strains of herpesvirus.

Excess dye was removed by cation exchange resin (Dowex 50-WX4). Tissue cultures containing virus alone served as controls. All three sets of cultures were titrated for infectivity in amnion cells after incubation at 37° for 7 days. Those that exhibited cytopathic changes after inoculation of a virus–dye mixture were passed once or twice in amnion cell cultures. After full infectivity was established, they were harvested and subjected to repeated study as outlined above until resistance to photoinactivation by neutral red, methylene blue or a mixture of both became apparent.

Results. In vitro photoinactivation of herpesvirus. The degree of photosensitivity of herpes virus to proflavine, neutral red and methylene blue was defined as follows: *Highly photosensitive*—no change of infectivity in the dark, but complete inactivation of virus in the presence of light. *Moderately sensitive* —greater than 100-fold reduction of viral infectivity after light exposure. *Least sensitive*—less than 100-fold reduction of viral titers in the light, as compared to the dark. Activity in dark was defined as a reduction of infectivity by more than 100-fold without exposure to light.

Thirty-seven strains of herpesvirus were examined for photosensitivity to methylene blue (Table I). Of these, 27 were highly and four moderately sensitive; six were least sensitive. Neutral red produced almost identical effects; two strains *moderately* sensitive to one dye were *highly* susceptible to the other. Cross-insensitivity was complete. Proflavine, on the other hand, behaved very differently. Unlike methylene blue or neutral red, it was active against 14 or 22 strains of the virus; 12 were completely inactivated. Two strains were very sensitive to proflavine; 2 were least sensitive to this dye but moderatelv sensitive to the others.

Photosensitivity to combined dyes. Twenty-eight strains of herpesvirus were examined for photosensitivity to a combination of neutral red and methylene blue as well as to each dye alone. The mixture of the dyes did not increase the sensitivity of most of the strains susceptible to the individual agents; however, four strains which were moderately sensitive to one or the other dyes alone became highly susceptible when treated with both simultaneously. Strains least sensitive to methylene blue or neutral red were no more susceptible when exposed to these agents at the same time.

Development of resistance. Of 20 photosensitive strains repeatedly cultured in the presence of methylene blue, neutral red or a combination of both *in vitro*, 16 became resistant after one and the rest after two passages. The combination of both dyes did not prevent the development of resistance.

Discussion. Methylene blue, tetramethylthionine is a phenothiazine derivative closely related chemically to neutral red, a phenozine compound, but differs from proflavine which is a congener of acridine. This may account for the similarity or difference in their photosensitizing activity.

Seventy-three percent of strains of herpesvirus were found to be totally inactivated by exposure *in vitro* to methylene blue and light in the present study; 16% were least sensitive. The antiviral effect was not produced in the dark, but was maximal when exposure to light was added. Almost identical results

were produced by neutral red. Proflavine, unlike the other dyes, was active in the dark for more than half of the strains studied. The incidence of insensitivity to this agent was less than that for methylene blue or neutral red. Strains with low sensitivity to proflavine were sensitive to the two other agents in the presence of light. Because the degree of photosensitivity produced by methylene blue and neutral red was approximately the same, it was not surprising to find that come bining them did not increase photosensitivity or prevent the development of resistance. Despite this, resistance to each agent alone or to a combination of them developed rapidly *in vitro*. These *in vitro* observations indicate that the clinical application of light-dye therapy with either methylene blue or neutral red will be most likely unsuccessful if the strains of virus involved are only slightly sensitive to these agents in the presence of light *in vitro*.

The mechanism of varying degrees of viral sensitivity and induced viral resistance to photoinactivation is not clear. It has been demonstrated that the dye combines with the guanine base portion of nucleic acid and that, on light exposure, deletion of the dye-guanine complex occurs (8, 9). Resistance may be associated with the failure of either complex formation or subsequent deletion.

Although methylene blue is not a vital dye, it enters living cells. (Unpublished observation). Methylene blue stains primarily the nucleus, but it does not persist in the cells when the medium of the cultured cells is kept free of the dye.

Conclusion. Methylene blue, in a concentraion of 10^{-5} *M* was virostatic in the presence of light but not in the dark for 31 of 37 strains of fresh isolates of herpesvirus hominis. Resistance to the dye developed during treatment. This photodynamic pattern was almost identical to that of neutral red which produced an identical effect. This was not true for proflavine which was active in the dark in many instances. Cross insensitivity between proflavine and methylene blue was not observed.

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Induction of Extra Nephrons in Unilaterally Nephrectomized Immature Rats[1] (38525)

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Contrary to prevailing opinion on the matter, it has recently been discovered (1, 2) that the number of glomeruli that develop in the rat kidney can be increased above normal levels by performing unilateral nephrectomies in young animals. It is well established that in the adult rat, compensatory renal hypertrophy is not accompanied by increases in the number of nephrons (3, 4). Even in young rats, earlier investigators (5–7) had concluded that the remaining kidney could develop no more nephrons than they normally do. Hence, recent reports to the contrary deserve to be confirmed and amplified to prepare the way for future investigations of the physiological factors controlling nephrogenesis.

Materials and Methods. Sprague Dawley rats (Charles River CD) of both sexes were used in these experiments. Kidneys were removed through dorsolateral incisions from newborn rats immobilized by a 30 min exposure to 5°, or from older ones anesthetized with ether.

In a total of 14 litters, averaging 10 per litter, some animals were sacrificed at 10-day intervals from birth to 50 days, as well as at 70 and 100 days, as a source of kidneys from which the normal growth and nephron number could be determined during maturation. The remaining littermates were unilaterally nephrectomized on the same schedule up to 70 days of age and their removed kidneys were combined with those of the unoperated controls. The experimental rats were all sacrificed at 70 days of age, except for the ones nephrectomized at 70 days, which were sacrificed when they were 100 days old.

Removed kidneys were decapsulated and weighed prior to being prepared for glomerular counts according to a modification of established procedures (2, 8). Such kidneys were macerated in 50% HCl at room temperature for 24 hr and stored for up to 2 days in distilled water at room temperature until counted. Each kidney was made into a suspension in 50 ml of water by gently drawing it in and out of the wide end of a pipette until its tissue fragments were completely separated. A 0.1 ml sample of this suspension was drawn into a syringe and placed in a counting chamber. The sample was suspended freely between a microscope slide and cover glass at a depth of 1 mm without making contact with the edges of the chamber in order to avoid undue distortion. Glomeruli were then counted at 40X magnification, being made visible by the orange color of the residual blood they contained. Two samples of each kidney were averaged, and if their counts varied by more than 5%, a third sample was taken and all of them were averaged for a final estimate. The glomerular counts from such samples were then extrapolated to give a total glomerular count per kidney.

Results: A comparison of Figs. 1 and 2 reveal that although the absolute weight of the kidney continues to increase during the first 100 days of life, its relative weight begins to decline after the 10th day. Thus, the most rapid growth of the kidney occurs during the first 10 days after birth at which time they are increasing in mass at a rate greater than that of the body as a whole.

It is during this period that the number of glomeruli increases most rapidly. In agreement with earlier investigators (9, 10) we find that the newborn kidney contains approximately 10^4 nephrons. This number is more than doubled by 10 days of age and the adult complement of $34–35 \times 10^3$ nephrons is reached by 40 days (Fig. 3).

When one kidney is removed from the newborn rat, the remaining kidney at 70

[1] Supported by U.S. Public Health Service Grants Nos. HL-13659 and HL-16336.

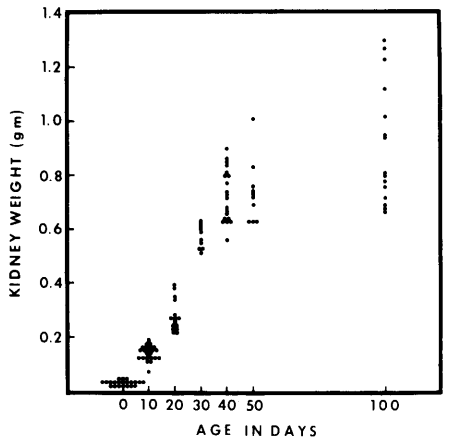

FIG. 1. Increase in absolute weight of rat kidneys as a function of age.

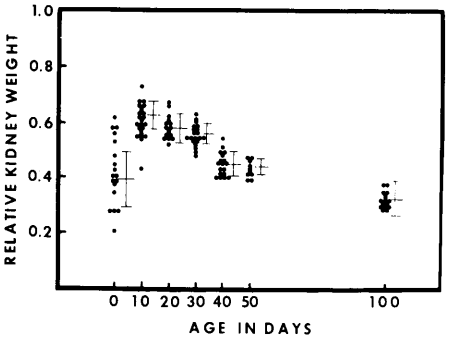

FIG. 2. Relative kidney weights [(kidney wt/body wt) × 100] in rats at different ages, including means ± SE.

days of age is found to contain approximately 57 × 10^3 glomeruli, representing a 63% increase over control values. Figure 4 illustrates how this increase in the complement of glomeruli is influenced by unilateral nephrectomies performed in progressively older rats. Up to the age of approximately 50 days their capacity to augment the adult population of nephrons is gradually abridged. Unilaterally nephrectomized 70-day old rats react as adults in that compensatory renal hypertrophy is accompanied by no further increase in nephron number.

Discussion. Normal kidney growth in the rat is achieved predominantly by cellular proliferation during the early stages of development and by cell hypertrophy at progressively later stages (11). At the tissue level of organization, the infant kidney continues to make new nephrons up to approximately 40 days of age, a process which cannot go on without the addition of new cells. Occurring principally in the outer

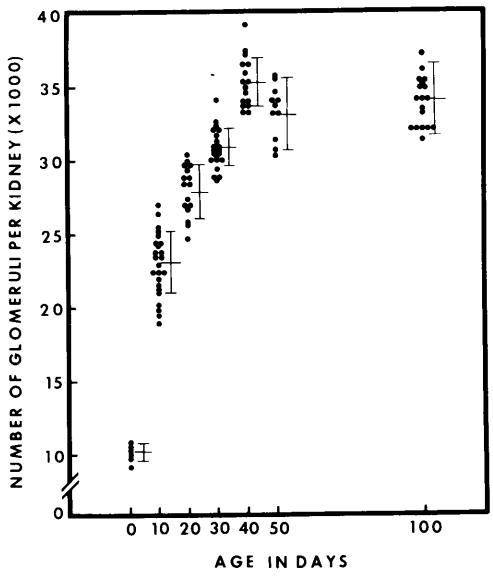

FIG. 3. Normal increase with age in the number of nephrons per kidney based on glomerular counts.

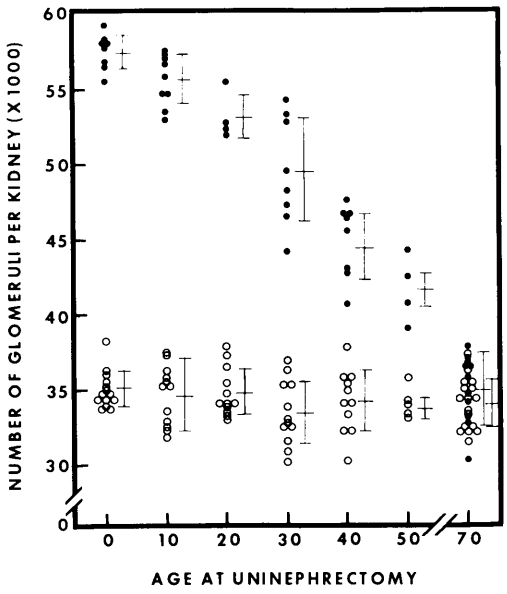

FIG. 4. Distributions of glomerular counts (solid dots) in rats unilaterally nephrectomized at different ages and sacrificed at 70 days. (Those operated at 70 days of age were sacrificed at 100 days.) Open circles indicate control values for unoperated littermates at 70 or 100 days, respectively.

nephrogenetic zone (12) the differentiation of nephrons in the developing kidney gradually decreases until the adult complement is attained. It is interesting to note, however, that even after this point there is approximately a 10 day period when the kidney is capable of increasing its population of nephrons above normal even though it does not ordinarily do so. Hence, unilateral nephrectomy of a 50-day old rat can still elicit a 20% increase in the number of glomeruli by 70 days of age. Thus, the potential for nephrogenesis seems to persist for a short time after the last nephron is produced.

In view of the present confirmation of the discovery by Bonvalet *et al.* (1) and Imbert *et al.* (2) that immature rat kidneys are in fact capable of making extra glomeruli, it is difficult to explain how others (5–7) failed to detect such a response in rats unilaterally nephrectomized at various ages from birth to 50 days. On the other hand, one would expect to find supernumerary nephrons in cases of the congenital absence of one kidney. According to Moore (13), this was indeed the case in a human subject, but Boycott (14) found no such result in a rabbit born with only one kidney, despite the fact that the remaining one had undergone considerable hypertrophy by the time it was examined in the adult.

The fact that compensatory growth of the kidney in immature rats is accompanied by the production of extra numbers of nephrons suggests that the species-specific complement of nephrons is not genetically determined. This possibility demands the search for physiological factors in the developing organism to which the production of nephrons adapts. In fishes, normally capable of adding new nephrons throughout life (15), there is evidence that the salinity of the water in which they are raised is inversely proportional to the number of glomeruli that develop in their kidneys (16, 17). Growth retardation in mice by hypoxia (18) or in rats by maternal malnutrition (19) also reduces the number of glomeruli per kidney. Clearly, the time is at hand to contemplate experiments designed to test a variety of physiological parameters for their ability to influence the number of nephrons that develop in the growing animal.

Summary. The normal number of glomeruli per kidney in the rat rises from about 10^4 at birth to approximately 35×10^3 at 50 days of age. When one kidney is removed at birth the remaining one produces an average of 63% more nephrons than normal by 70 days. Unilateral nephrectomy of successively older rats results in progressively less augmentation of the nephron complement in the remaining kidney up to 50 days, beyond which age the kidney loses its ability to produce new nephrons.

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Human Adipose Tissue Lipoprotein Lipase: Comparison of Assay Methods and Expressions of Activity[1] (38526)

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(Introduced by Edwin L. Bierman)

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Lipoprotein lipase (LPL), an important enzyme in the peripheral uptake of triglycerides (Tg) from plasma (1), has been measured in animal (2, 3), and more recently, in human (4, 5) adipose tissue. LPL from animal tissues usually has been assayed by one of two ways: as heparin releasable enzymatic activity from intact cells or as the activity in NH_4OH extracts of acetone–ether powder of adipose tissue. The former has been thought to correlate with postheparin lipolytic activity, the latter to represent the total enzymatic activity in adipose tissue (6). The purpose of the present communication is to compare these two techniques for assessment of enzymatic activity in adipose tissue of normal man. Since the degree of obesity varies considerably among humans and the LPL activity found may be dependent on the method of expression of activity, the activities were calculated per unit weight of adipose tissue and per fat cell for comparison.

Materials and Methods. Eight normal males, aged from 29–47 yr, were used in the study. Adipose tissue aspiration biopsies from subcutaneous buttock fat were performed by the technique of Hirsch *et al.* (7) after an overnight fast (12 hr). The tissue yield was usually 100–120 mg. After rinsing in approximately 100 ml of Krebs–Ringer phosphate (KRP) buffer (pH 7.4), tissue pieces were divided into three parts. One piece (5–10 mg) was used for measurement of fat cell diameter from a formaldehyde fixed, frozen section utilizing the method of Sjöström *et al.* (8). Fat cell size and the number of fat cells per gram of tissue were calculated from the fat cell diameter according to Goldrick (9). The two pieces for the LPL assays (40–50 mg each) were dried on Whatman filter paper and then weighed.

For the measurement of heparin releasable LPL, adipose tissue pieces were incubated for 45 min in a Dubnoff metabolic shaker at 37° in 2.5 ml of KRP buffer containing 2 unit/ml of heparin. Thereafter two 1.0 ml samples were taken for assay. The other tissue pieces were used to prepare an acetone–ether powder according to Nilsson-Ehle *et al.* (10). The acetone–ether powders were then homogenized in 0.4 ml of 0.05 *M* NH_4OH (pH 8.2) containing 0.15 *M* of NaCl, and the 0.4 ml samples used for assay. In separate experiments, acetone–ether powder was made also from tissue pieces, after they were first incubated with heparin and then rinsed in KRP buffer, to evaluate that amount of the total LPL activity of the tissue which is heparin releasable.

The substrate for LPL assay was prepared as follows: 100 μl of unlabeled triolein (25 mg/ml in benzene); 0.5 ml of 1-^{14}C-triolein (2 μCi/ml in benzene) and 10 μl of purified egg lecithin (12 mg/ml in chloroform–methanol, 1:1) were evaporated with nitrogen. This was then emulsified in 2 ml of a mixture of 10% fatty acid free bovine serum albumin (pH 8.0), normal human plasma, 2 *M* Tris–HCl buffer and distilled water (4:1.5:5:5:9.5) for 3 min using Branson 125 Sonifier (setting 3 and maximal tuning). The tube was kept in ice during and after sonification.

For lipoproprotein lipase assay 1.0 ml of incubation medium or 0.4 ml of NH_4OH homogenate of acetone–ether powder, and 0.2 ml of substrate were incubated for 30–60 min at 37° in Dubnoff metabolic shaker. The reaction was terminated by adding 10 ml of

[1] This study was partially supported by NIH project Grant No. AM 06670 and training Grant No. AM 05498. The travel of O.J.P. to U.S.A. was partially supported also by Duodecim Foundation, Helsinki, Finland.

Dole's extraction mixture. One ml of KRP or 0.4 ml of NH_4OH, and 0.2 ml of substrate, to which Dole's mixture was added was used as a control blank in the assay. Free fatty acids (FFA) were extracted as previously described (11). The enzymatic activity was calculated as nanoEq FFA released/min/g of tissue or per 10^6 cells.

The coefficient of variation (replicate analysis) for the heparin releasable activity within assays was 5.9%. This calculation could not be done for the extract of the acetone–ether powders of adipose tissue since they were not done in duplicate. To account for variability between assays a postheparin pooled plasma sample was run in each assay: 10 μl of postheparin plasma and 0.2 ml of the substrate were incubated and free fatty acids extracted at the same time as the adipose tissue LPL. PHLA values usually differed less than 10%. On occasions when this was exceeded the results were corrected to the corresponding mean PHLA values.

The percent of ideal body weight was calculated from the mean ideal body weight for height obtained from the Metropolitan Life Insurance tables.

Results and Discussion. LPL activities from heparin incubated adipose tissue and acetone–ether powder of adipose tissue were expressed as activity per gram of adipose tissue or per 10^6 cells of adipose tissue (Table I). Heparin releasable enzymatic activity is about two times higher than the activity in acetone–ether powder, and there is a significant positive correlation between these activities ($r = 0.92$, $P < .01$; Fig. 1). This result would be unexpected, if LPL of acetone–ether powder represents the total enzymatic activity, and only a part of that activity can be released by heparin, as has been previously suggested (6). However, in studies with rat adipose tissue (11, 12), these two enzymatic activities were similar, and in the study of Stewart and Schotz (13), the "total" LPL activity of rat adipose tissue (measured from buffer homogenate) was only one fifth of that activity released into the medium with heparin. One explanation for this apparent discrepancy could be the activation of LPL during release process (13), so that the activity in the heparin extract may be higher even if only a part of the originally inactive enzyme is released. On the other hand, only a part of the total LPL activity of acetone–ether powder might be extractable with the conventional methods (14). That one of the above possibilities exists, is supported by the results of the present study: Of the original LPL activity 44–70% remained in acetone powder of adipose tissue after preincubation with heparin (Table II). These results fit quite well with studies in animals, in which adipose tissue LPL activity decreased 20–30% (6) or 70–80% (15), after *in vivo* heparin injection. However, such studies should be interpreted cautiously, because even after rinsing, some LPL activity released and activated by heparin may be left in the tissue.

The subjects used in this study were selected to provide a wide range of adult-

TABLE I. ADIPOSE TISSUE LPL IN EIGHT NORMAL SUBJECTS.[a]

Subject	Heparin releasable LPL		LPL of acetone–ether powder	
	g	10^6 cells	g	10^6 cells
E.B.	16.5	7.9	9.8	4.7
J.B.	11.2	4.7	3.9	1.6
R.C.	5.4	1.8	2.2	0.8
G.L.	5.1	2.8	2.1	1.2
O.P.	3.4	1.4	1.7	0.7
P.R.	8.6	3.2	4.2	1.6
P.S.	6.3	4.0	3.6	2.3
S.W.	5.2	1.6	1.3	0.4
Mean ± SD	7.7 ± 4.0	3.4 ± 2.0	3.6 ± 2.5	1.7 ± 1.3

[a] The activities are expressed as nanoeq FFA liberated/min/g of adipose tissue or 10^6 fat cells.

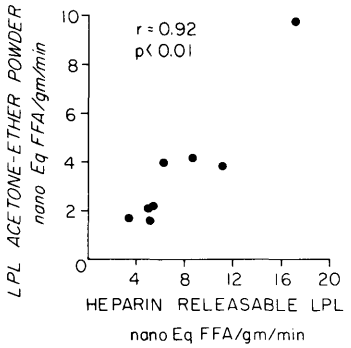

FIG. 1. Relationship between heparin releasable LPL and acetone–ether powder LPL in human adipose tissue.

TABLE II. THE LPL ACTIVITY IN ACETONE–ETHER POWDER BEFORE AND AFTER INCUBATION OF TISSUE PIECES WITH HEPARIN.[a]

	A	B	
Subject No.	LPL in acetone powder	LPL in acetone powder after heparin extraction	B/A × 100
1	2.12	1.37	64.6%
2	3.03	2.12	69.9%
3	2.32	1.01	43.5%
4	2.35	2.17	64.7%

[a] The activities are expressed as nanoEq FFA/min/gm of adipose tissue.

onset obesity. It has been suggested that in this type of obesity fat cell number approximates normal while fat cell size is increased (16, 17). The results of this study support this concept, since there is a positive correlation between obesity (expressed as percent ideal body weight) and fat cell diameter ($r = 0.91$, $P < .01$; Fig. 2A) and an inverse correlation between obesity and fat cell number per unit weight of adipose tissue ($r = -0.89$, $P < .01$; Fig. 2B). Thus, the LPL activity per unit weight and per fat cell would be quite different among these subjects; the more obese the person is, the lower the enzymatic activity per unit weight of tissue as compared to the activity per fat cell.

The results of this investigation demonstrate that the method of expression of the LPL activity may be important in comparative clinical studies. Many diseases, e.g., diabetes and endogenous hypertriglyceridemia, are frequently associated with obesity. If subjects are not weight-matched, low enzymatic activity per unit weight may then lead to an erroneous conclusion even though LPL activity per total body fat would be normal. Since fat cell size, but not fat cell number, increases in adult-onset obesity, the ratio between total body fat and fat cell size remains the same. Therefore, if LPL activity is expressed per fat cell, the influence of obesity can be avoided.

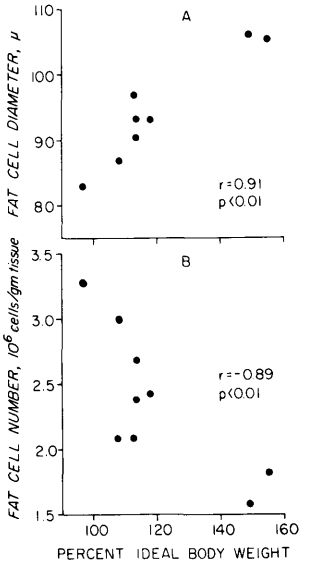

FIG. 2. Relationship between ideal body weight and fat cell diameter (A) and fat cell number per gram of adipose tissue (B) in normal subjects.

Summary. There was a positive correlation in normal man between heparin releasable lipoprotein lipase and lipoprotein lipase of ammonium hydroxide homogenate of acetone–ether powder in adipose tissue. Heparin releasable as lipoprotein lipase activity was about twice as high as the enzymatic activity in acetone powder, even though 40–70% of the original activity remained in the tissue after incubation with heparin. This might indicate that activation of the enzyme is associated with its release by heparin from tissue. The lipoprotein lipase activity per unit weight and per fat cell were affected differently by obesity: In obese subjects lipoprotein lipase per unit weight was proportionally lower than the activity per fat cell. The expression of

activity per fat cell appears to avoid the effect of obesity, and hence increased fat cell size, on values obtained.

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Chemotactic Deactivation of Human Eosinophils by the Eosinophil Chemotactic Factor of Anaphylaxis[1] (38527)

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The eosinophil chemotactic factor of anaphylaxis (ECF-A) is an acidic peptide (1) mediator of approximately 500 mol wt which is preferentially chemotactic for guinea pig (2, 3) and human (1, 4, 5) eosinophils as assessed with mixed populations of target cells, and is the most active eosinophilotactic factor for purified eosinophils when compared to other factors at concentrations exhibiting similar neutrophil chemotactic activity (1). ECF-A is released by antigen challenge from guinea pig lung fragments passively sensitized with IgG1 (2), and by an IgE dependent mechanism from human lung and nasal polyp fragments (4, 6, 7), isolated human lung cells (8) and purified rat peritoneal mast cells (6). ECF-A is a preformed mediator which has been specifically associated with rat mast cells (6) and human leukemic basophils (9) by its extraction from essentially pure populations of these cell types; in the rat mast cell it is associated with the granules (6).

Rabbit neutrophils preincubated with certain chemotactic factors exhibit a time-and-dose-dependent diminution of the chemotactic response to a subsequent stimulus; this phenomenon has been termed deactivation (10). Deactivation is associated with the activation and decay of an esterase required for the chemotactic response and derived from proesterase one by stimulation with the chemotactic principle (11). In contrast to complement-derived factors, low molecular weight bacterial chemotactic factor neither deactivates the cell nor activates proesterase one (12). In the present study the capacity of human eosinophils to undergo deactivation in response to ECF-A and other principles has been established, and deactivation by ECF-A has been further analyzed for its time course, selectivity and magnitude.

Materials and Methods. Polystyrene disposable chemotactic chambers (Adaps, Inc., Dedham, MA) were assembled with 3 μm or 8 μm pore size micropore filters (Millipore Corp., Bedford, MA) as previously described (6). Hanks' solution and Medium 199 with phenol red (Microbiological Associates, Bethesda, MD), ovalbumin five times recrystallized (Miles Laboratories, Inc., Miles Research Co., Kankakee, IL), dextran, Sephadex and Ficoll (Pharmacia Fine Chemicals, Inc., Piscataway, NJ), sodium diatrizoate (Hypaque, Worthington Laboratories, New York, NY), sodium metrizoate (Triosil-75, Glaxo, Ltd., England), two times recrystallized trypsin and soybean trypsin inhibitor (Worthington Biochemical Corp., Freehold, NJ), (1-^{14}C)-glucose (Amersham-Searle Corp., Arlington Heights, IL), sodium lauryl sulfate (SLS) (Fisher Scientific Co., Medford, MA) and plastic 35 × 10 mm Petri dishes (Falcon Plastics, Div. of B–L Laboratories. Inc., Los Angeles, CA) were obtained from the manufacturers.

Beta radiation from (^{14}C) glucose solution was quantitated with Bray's fluid in a liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, IL).

Measurement of chemotaxis. Purified populations of eosinophils, mononuclear leukocytes and neutrophils were obtained from peripheral blood of normal donors or individuals with eosinophilia by modifications (6, 13) of previously published methods (14, 15). Chemotaxis of human leukocytes was assayed by a modification (6, 13) of the Boyden micropore filter assay (16) employing 3 μm filters for neutrophils (13) and eosinophils (6), and 8 μm filters for mono-

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nuclear leukocytes (16). The medium for all experiments was Hanks' balanced salt solution, 0.5% ovalbumin, pH 7.4. The chemotactic factors in all experiments were ECF-A purified from extracts of human lung by Sephadex G-25 gel filtration at a final concentration of 2 g equivalents of lung/ml (6), human C5a generated by tryptic digestion of 50 μg highly purified C5/ml (17) and human kallikrein generated from purified prekallikrein by activation with Hageman factor fragments and generating 2.5 μg bradykinin/ml from 0.2 ml heat inactivated plasma (18). 2×10^6 cells were incubated for 3 hr at 37° in moist chambers and the leukocyte responses were expressed as the mean of 10 high power fields (hpf), five from each of duplicate chambers, corrected for background counts in filters from chambers without a chemotactic stimulus.

Deactivation. Human leukocytes were incubated at room temperature from 1 to 60 min with various chemotactic factors. The cells were then sedimented at room temperature by centrifugation for 5 min at 400 *g* and resuspended in buffer. The wash procedure was performed twice and the cells transferred to the cell compartment of a modified Boyden chamber where they were incubated for a further 3 hr with a chemotactic stimulus. Cells incubated with buffer rather than a chemotactic factor during the deactivation period served as controls. Net chemotaxis reflects a correction for spontaneous migration in buffer alone. Spontaneous migration varied from 0 to 5 cells/hpf for control cells to 0–8 cells/hpf for cells preincubated with chemotactic factors to achieve deactivation. The extent of deactivation was quantitated and expressed as:

chemotaxis (% of control)

= (net chemotaxis of deactivated cells/

net chemotaxis of control cells) × 100

Hexose monophosphate shunt (HMPS) activity of adherent leukocytes. The HMPS activity of purified adherent neutrophils and eosinophils was determined by measuring the extent of conversion of (1-^{14}C) glucose to $^{14}CO_2$ after 80 min at 37° (19). The cpm of $^{14}CO_2$ were standardized by dividing each value by the OD at 280 nm of a 3% SLS solution of the adherent leukocytes and the results expressed as cpm/0.2 absorbency units 280 (AU 280). The effect of chemotactic factors upon HMPS activity was calculated by subtraction of the mean value for duplicate control cells from that of treated cells.

Results. Chemotactic deactivation by ECF-A of human eosinophils. The capacity of human eosinophils preincubated with ECF-A to be rendered unresponsive to subsequent stimulation by the same principle in a chemotactic chamber was analyzed with both purified and unpurified cells. Eosinophils were purified to 77% purity from a donor (M.S.) with a drug reaction, while those from a patient with rheumatoid arthritis (G.R.) with 90% eosinophilia were employed unpurified. 2×10^6 eosinophils were exposed for varying times to a 1:10 dilution of ECF-A and compared to identical populations of eosinophils preincubated for the same duration with buffer alone. The chemotactic response of cells from these two individuals preincubated with ECF-A was diminished at 2 min by 70 and 80%, respectively (Fig. 1).

The effect of varying concentrations of ECF-A on deactivation of 2×10^6 eosinophils purified to 83% purity from a patient (H.B.) with metastatic carcinoma was compared at 2 and 30 min. Incubation of 2×10^6 eosinophils for 30 min with dilutions of ECF-A ranging from 1:10 to 1:160 resulted in nearly complete deactivation. At 2 min deactivation was dose-dependent, with a

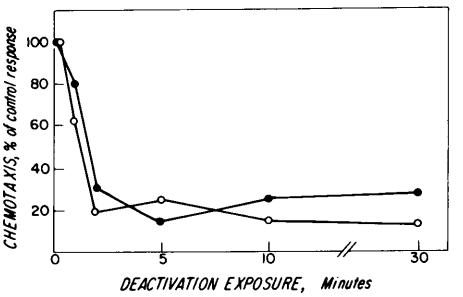

FIG. 1. Time course of chemotactic deactivation of human eosinophils by ECF-A. A 1:10 dilution of ECF-A attracted 49 control eosinophils from patient M.S. (●——●) and 78 from patient G.R. (○——○). Deactivation is depicted as % control chemotactic response.

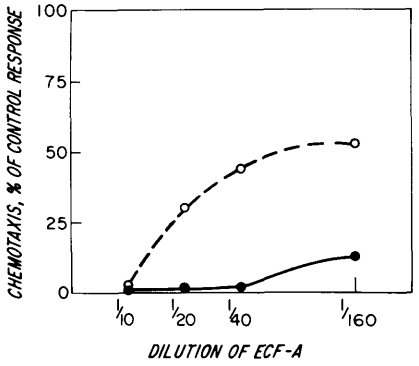

FIG. 2. Dose response of chemotactic deactivation of human eosinophils by ECF-A. A 1:10 dilution of ECF-A attracted 55 control eosinophils/hpf. Deactivation is depicted as % control response of cells exposed to ECF-A for 2 min (○——○) and 30 min (●——●).

50% reduction in chemotactic response occurring at a 1:40 dilution (Fig. 2). As the net chemotactic activity, assessed at 3 hr in a modified Boyden chamber, of the dilutions employed was 55, 45, 28 and 3 cells/hpf for the dilutions 1:10, 1:20, 1:40 and 1:160, respectively, deactivation was apparent at both 2 and 30 min with a concentration (1:160) which was not demonstrably chemotactic.

The effect of preincubation with ECF-A on the responses of 2×10^6 eosinophils (88% purity, patient M.S.), neutrophils (93% purity), and mononuclear cells (96% purity), to ECF-A, kallikrein, and C5a, respectively, was determined. Each cell type was incubated with a 1:10 dilution of partially purified ECF-A for varying time intervals, washed twice and placed in a modified Boyden chamber for assessment of the chemotactic response. The fall in chemotactic activity of the mononuclear and neutrophilic leukocytes was 30% and 20%, respectively, at 2 min as compared to 72% for eosinophils, and there was little change thereafter (Fig. 3). The chemotactic activity of the ECF-A employed was 37 control eosinophils/hpf, 12 control mononuclear cells/hpf and 8 control neutrophils/hpf.

Chemotactic deactivation of eosinophils by diverse stimuli. 2×10^6 eosinophils of 72% purity from patient M.S. were exposed to dilutions of ECF-A (1:20) and C5a

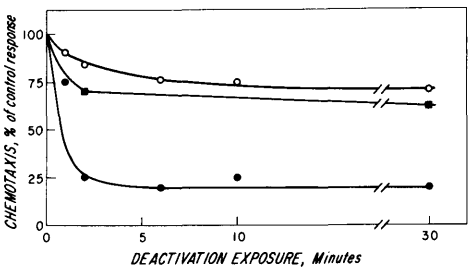

FIG. 3. Time course of deactivation of human leukocytes by ECF-A. Chemotactic stimuli were a 1:10 dilution of ECF-A which attracted 37 control eosinophils/hpf (●——●), a 1:10 dilution of C5a which attracted 56 control mononuclears/hpf (■——■) and a 1:10 dilution of kallikrein which attracted 63 control neutrophils/hpf (○——○). Deactivation is depicted as % control chemotactic response.

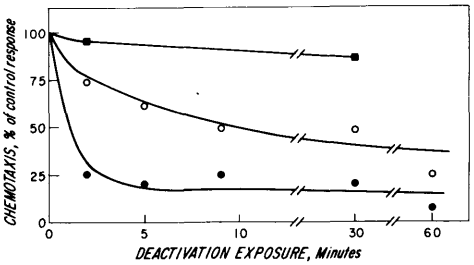

FIG. 4. Eosinophil deactivation by ECF-A (●——●), kallikrein (■——■) and C5a (○——○). Chemotactic response of control eosinophils to a 1:10 dilution of ECF-A was 21 cells/hpf. Deactivation is depicted as % control chemotactic response.

(1:10), which exhibited comparable chemotactic activity for eosinophils, and to a dilution of kallikrein (1:10) which exhibited chemotactic activity similar to C5a for purified neutrophils. After varying time intervals the cells were washed twice and transferred to modified Boyden chambers to assess chemotaxis to a 1:10 dilution of ECF-A. C5a elicited a slower and less profound deactivation of eosinophils than did ECF-A, while kallikrein had no demonstrable effect (Fig. 4).

Deactivation, which had been routinely assessed by diminution in response to ECF-A, was next examined by comparing ECF-A and C5a in a cross deactivation experiment. 2×10^6 eosinophils were exposed for 30 min to dilutions of ECF-A (1:40) and C5a (1:20) exhibiting comparable chemotactic activity for eosinophils or

to a dilution of kallikrein (1:10) which exhibited chemotactic activity similar to C5a for purified neutrophils. The cells were washed twice and portions were transferred to modified Boyden chambers to assess their chemotactic response to C5a and to ECF-A. As shown in Table I, eosinophils deactivated by ECF-A or by C5a were equally limited in their response to the homologous or heterologous stimulus, reflecting full cross deactivation. Kallikrein was not chemotactic for eosinophils and did not deactivate them for either stimulus.

The interaction between chemotactic factors and adherent neutrophils is associated with a stimulation of HMPS activity as assessed by generation of radioactive $^{14}CO_2$ from ($^{14}C_1$) glucose (19). Thus C5a (1:40) and kallikrein (1:40) exhibiting chemotactic activity for purified neutrophils from a normal donor demonstrated augmentation in HMPS activity of these cells; while ECF-A (1:25), at the concentrations employed, lacked chemotactic activity for this human neutrophil population, and caused only a minimal augmentation in HMPS activity. The effect of these same factors upon the HMPS of adherent eosinophils was also examined. Both ECF-A and C5a, with eosinophilotactic properties, increased HMPS activity; while kallikrein was minimally active (Table II).

TABLE I. CHEMOTACTIC CROSS DEACTIVATION OF HUMAN EOSINOPHILS.

	Chemotactic stimulus % deactivation at 30 min	
Deactivating agent	ECF-A	C5a
ECF-A	90	90
C5a	75	70
Kallikrein	<10	<10

Discussion. Deactivation, the time- and dose-dependent diminution of the chemotactic response of neutrophilic leukocytes, rabbit (10) or human (20), induced by preincubation with chemotactic principles is also a characteristic of the eosinophil. At 2 min deactivation of eosinophils by ECF-A could be shown to be dose-dependent, while complete deactivation was noted with a range of doses over a 30 min period (Fig. 2). The deactivation of eosinophils observed with a non-chemotactic dose of ECF-A (1:160, Fig. 2) may be due to the concentration or distribution of ECF-A at the surface of all the cells as compared to the relationships achieved by diffusion of ECF-A through the micropore filter of the chemotactic chamber. Deactivation by ECF-A was demonstrable not only with purified eosinophils but also with eosinophils obtained directly from a patient with 90% eosinophilia (Fig. 1). A structurally unrelated eosinophilotactic factor, C5a, employed at a comparable eosinophilotactic dose also was capable of eliciting deactivation (Fig. 4). Further, there was cross deactivation between ECF-A and C5a, while kallikrein, which was not eosinophilotactic at the concentrations employed, did not deactivate eosinophils to either of the aforementioned stimuli (Table I).

A relationship between chemotaxis and deactivation has been observed both in the relative deactivating activity of diverse chemotactic stimuli on the same cell type, and in the response of different cell types to the same stimulus. Thus ECF-A and C5a,

TABLE II. EFFECT OF CHEMOTACTIC FACTORS ON HMPS ACTIVITY OF HUMAN EOSINOPHILS AND NEUTROPHILS.

	HMPS activity net cpm/0.2 AU_{280}[a]		Chemotaxis net cells/hpf	
Eosinophils	Eosinophils	Neutrophils	Eosinophils	Neutrophils
ECF-A	554	290	6	0
C5a	474	1014	10	27
Kallikrein	154	686	1	19

[a] Reflects correction for the baseline values of control neutrophils and eosinophils which had 897 and 754 cpm/0.2 AU_{280}, respectively.

which are eosinophilotactic, deactivated eosinophils, while kallikrein did not (Fig. 4). On the other hand, kallikrein, which is chemotactic for neutrophils, deactivated this cell type even more rapidly than a comparably chemotactic dose of C5a (20). Further, the modest deactivation of mononuclear cells and neutrophils by a dose of ECF-A which profoundly deactivates eosinophils is consistent with the relatively lesser chemotactic activity of ECF-A for these cells (Fig. 3) (21).

The stimulation of the HMPS of purified adherent neutrophils by chemotactic principles is dependent upon the functional integrity of the principle and is not observed with prekallikrein or kallikrein inactivated by diisopropylfluorophosphate (19). Stimulation of HMPS activity of purified adherent eosinophils was observed with ECF-A and C5a while kallikrein had a minimal effect (Table II). Conversely both kallikrein and C5a, which are chemotactic for neutrophils, stimulate their HMPS activity while ECF-A had a minimal effect compatible with its limited chemotactic activity for this cell type (Table II) (21).

The finding that deactivation follows interaction of rabbit or human leukocytes with chemotactic principles derived from human plasma such as kallikrein and C5a, or derived from mast cells (ECF-A) may imply a trapping mechanism for migrating cells. Thus cells attracted to a site of chemotactic factor generation might remain after the initial gradient had subsided or shifted elsewhere. The release of ECF-A by mast cells participating in IgE-dependent reactions would selectively attract and then hold eosinophils at the site of the reaction. A recently recognized regulatory function which could then be expressed by the eosinophil would be the release of arylsulfatase, possibly consequent to phagocytosis (1, 22) of mast cell granules (23) or the action of ECF-A itself, with consequent inactivation of SRS-A (22, 24).

Summary. Purified human eosinophils demonstrate diminished chemotactic responsiveness (deactivation) after incubation with the eosinophil chemotactic factor of anaphylaxis (ECF-A). The deactivation is rapid, and selective in that ECF-A deactivated human eosinophils more markedly than neutrophilic or mononuclear leukocytes. The eosinophil can also be deactivated by C5a which is eosinophilotactic, and there is cross deactivation between C5a and ECF-A. Deactivation may be an important physiologic control mechanism enabling the eosinophil to remain at sites of ECF-A release in order to manifest its regulatory functions in immediate hypersensitivity reactions.

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Effect of Respiratory and Metabolic Acidosis on Coronary Vascular Resistance[1] (38528)

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An increase in coronary flow has been observed during acute hypercapnia (1–4). On the other hand, metabolic acidosis has been shown to produce an increase (5), or a decrease (2, 6) in coronary vascular resistance. Similarly inconsistent results have been observed when either respiratory or metabolic alkalosis was produced (2, 4–6). The apparent contradiction between different studies may be due to one or more of the following. In the first place, changes in the coronary circulation were observed in the presence of changes in the systemic circulation, notably arterial blood pressure and heart rate, making it difficult to determine to what extent coronary changes are a reflection of systemic circulatory changes (1, 5, 6). Secondly, acid-base changes are capable of altering the O_2 requirements of the heart which in turn strongly affect the coronary circulation. This variable has not always been taken into account in the interpretation of the results (2, 4, 6). Finally, in some cases simultaneous changes in pH, P_{CO_2} and HCO_3^- concentration have thwarted attempts to ascertain which of these parameters is responsible for the changes observed (5, 6).

The present experiments were undertaken to study the effect of metabolic and respiratory acidosis on coronary vascular resistance. A preparation where hemodynamic variables and O_2 requirements could either be controlled or their influence accounted for was employed.

Methods. The experiments were performed using isolated perfused rabbit hearts. Rabbits were anesthetized with a mixture of chloralose and urethane. The thorax was opened under artificial ventilation and a metal cannula was introduced into the aorta, with its tip oriented towards the ventricle and close to the valve. Perfusion of the coronary circulation with Ringer's solution, equilibrated at 37° with 95% O_2, 5% CO_2 was started immediately with a roller pump. Coronary venous effluent was collected through a cannula in the pulmonary artery. Perfusion pressure was measured with a transducer connected to the aortic cannula. A wide bore needle was introduced into the left ventricle through the apex and connected to another transducer to record left ventricular pressure. The left ventricle was maintained empty by a catheter introduced in the cavity and connected to a small pump. Adequate Thebesian drainage was checked by the level of left ventricular pressure that was always equal to or lower than atmospheric. Heart rate was maintained constant by atrial pacing. Coronary venous flow from the pulmonary artery was measured by timed collection. P_{O_2} of the coronary venous effluent was continuously measured with a flow-through electrode. Perfusion pressure, left ventricular pressure and venous P_{O_2} were continuously recorded on a polygraph. Samples of arterial and venous perfusate were analyzed in triplicate for pH, P_{O_2} and P_{CO_2} with appropriate electrodes. Pressure-flow curves of the coronary circulation were obtained by changing the flow of the perfusion pump. Each curve consisted of at least four different flows. Arterial and venous perfusate samples for the determination of pH, P_{CO_2} and P_{O_2} were obtained at every flow level when a steady state was reached.

Three series of experiments were performed. In the first series, pressure flow curves were obtained during perfusion of the coronary circulation with control Ringer's solution (pH_a = 7.47 ± 0.01, P_aCO_2 = 34.6 ± 0.5 mmHg) and during perfusion with hypercapnic Ringer's solution (pH_a = 6.87 ± 0.004, P_aCO_2 = 141.8 ± 1.7 mmHg). In the second series of experiments, the

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effect of metabolic acidosis was studied. Pressure flow curves obtained during perfusion with control solution (pH_a = 7.43 ± 0.02, P_aCO_2 = 30.6 ± 0.9 mmHg) were compared with those obtained during perfusion with low HCO_3^- solution (pH_a = 6.98 ± 0.03, P_aCO_2 = 30.7 ± 1.0 mmHg). In order to avoid the influence of time, the hearts were perfused with different flows and different solutions in a random sequence. In a third group of five experiments, coronary flow was set at approximately 2.5 ml × min^{-1} × g^{-1}, and maintained unchanged throughout the experiment. The coronary circulation was perfused in a random sequence with control solution, hypercapnic solution and low HCO_3^- solution. The same variables as in the previous two groups were measured. In preparing the solution, special care was taken to attain the same pH with respiratory and metabolic acidosis.

Results. The results of the first series of experiments are shown in Fig. 1. In panel A, perfusion pressure values are plotted as a function of coronary flow for the control and hypercapnic solutions. No significant changes were observed during respiratory acidosis as compared to control; that is, for any given flow perfusion pressure was essentially the same in both conditions. Panel B shows the values of venous P_{O_2} for the same groups of experiments, plotted as a function of flow. A difference between control and acidosis is now evident. For any given flow, venous P_{O_2} was lower in control than in acidosis. Panel C shows a plot of perfusion pressure as a function of Pv_{O_2}. For any given Pv_{O_2}, perfusion pressure was substantially higher for the control experiments.

Essentially the same results were obtained when metabolic acidosis was produced (Fig. 2). A plot of perfusion pressure as a function of flow fails to show any difference between perfusion with control or low HCO_3^- solutions (panel A). On the other hand, when Pv_{O_2} is plotted as a function of flow, it can be seen that Pv_{O_2} was higher in acidosis for any given flow (panel B) and that for any given Pv_{O_2} perfusion pressure was lower during acidosis than control (panel C).

Table I shows the results obtained in the third series of experiments when both respiratory and metabolic acidosis were produced in the same preparation. Essentially the same venous pH was attained when P_{CO_2} was increased as when HCO_3^- was lowered. While flow was maintained unchanged induction of either type of acidosis did not significantly alter perfusion pressure. However, for a similar coronary resistance Pv_{O_2} was higher during acidosis than during control. No difference in Pv_{O_2} was seen between metabolic and respiratory acidosis.

Discussion. The preparation used in the present experiments had several features that made it specially suited for the study of the effect of acid-base alterations on the coronary circulation. By isolating the heart, neurohumoral and systemic circulatory influences were eliminated. Changes in extravascular compression were also avoided by keeping the left ventricle empty. Heart rate changes were eliminated by pacing the heart. pH could be changed by independently altering either HCO_3^- or P_{CO_2}. In addition, since arterial and venous P_{O_2} were measured at every point in the pressure flow curves, changes in myocardial O_2 consumption secondary to acidosis could be detected and their influence upon coronary vascular resistance taken into account.

No significant difference was observed between the pressure-flow curves of either respiratory or metabolic acidosis and their respective controls. In other words, for any given flow, perfusion pressure was essentially the same during perfusion with acid or control solution. These results indicate that, under this particular set of experimental conditions, acidosis did not alter the resistance to flow across the coronary vascular bed. Thus, it would be concluded that acidosis does not have a direct effect on the coronary vasculature. However, another explanation can be offered if the venous O_2 values are taken into consideration. It is well known that coronary vascular resistance is profoundly affected by changes in O_2 requirements (7, 8) and a relationship between $M\dot{V}O_2$, and coronary resistance has been amply demonstrated (9). When the coronary flow was increased in the present experiment, Pv_{O_2} increased both in control and acidosis, as it would be expected if $M\dot{V}O_2$ was not flow-limited. However, for any given flow, Pv_{O_2} was higher for acidosis

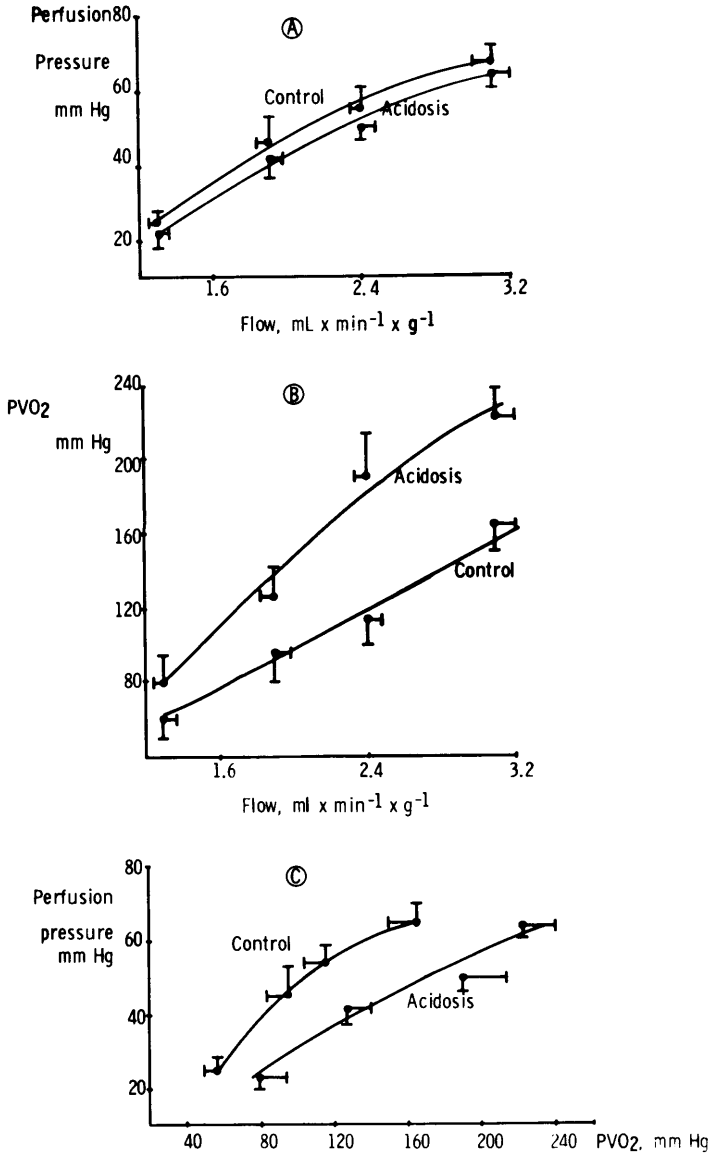

FIG. 1. Effect of respiratory acidosis on the coronary circulation. Panel A. Perfusion pressure is plotted as a function of coronary flow. In panel B, the P_{O_2} of the coronary effluent (P_{vO_2}) is plotted as a function of the corresponding coronary flow. Panel C shows the values of perfusion pressure of the same experiments plotted as a function of P_{vO_2}.

than for control (Figs. 1 and 2, panel B). The increase in Pv_{O_2} with acidosis for any given flow rate reflects a decrease in $M\dot{V}O_2$ probably secondary to the decrease in contractility that is known to accompany acidosis (2, 5, 10, 11). Also, for any given Pv_{O_2}, perfusion pressure is lower for acidosis than for control (Figs. 1 and 2, panel C). This indicates that, for any given level of Pv_{O_2}, the coronary resistance is lower during acidosis. If acidosis would have no effect whatsoever on coronary resistance it would be expected that, following a decrease in $M\dot{V}O_2$ associated with lowering pH, an increase in coronary resistance would occur. This was not the case, and the fact that acidosis was associated with a lower resistance to flow for any given Pv_{O_2} suggests that

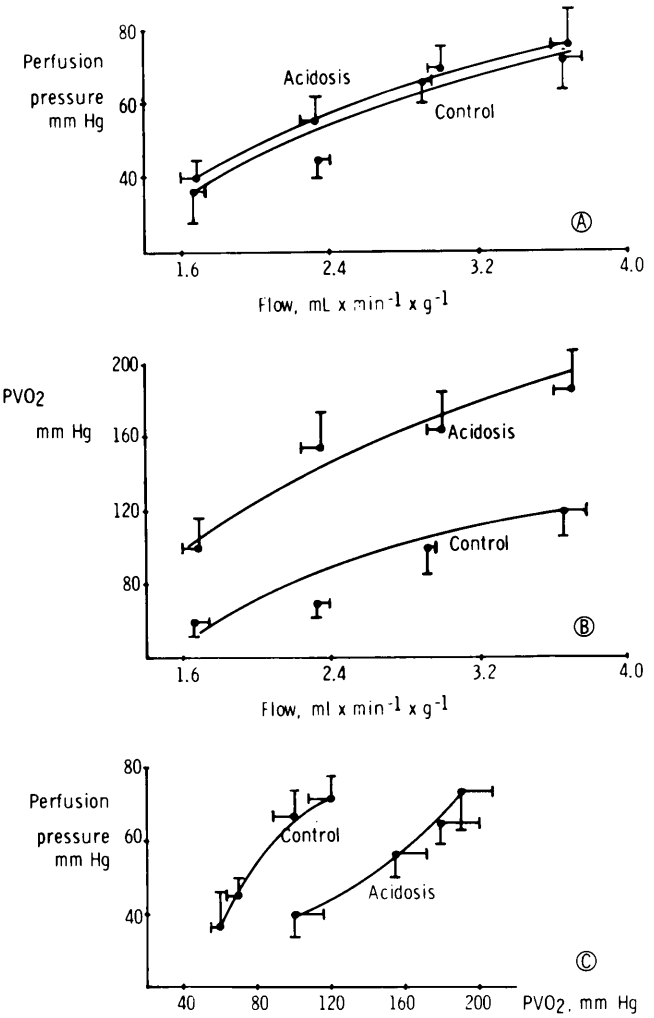

FIG. 2. Effect of metabolic acidosis on the coronary circulation. The results have been plotted in the same way as in Fig. 1.

TABLE I. RESULTS OF THE EXPERIMENTS OF GROUP III.[a]

	pH_v	P_{vCO_2} mm Hg	P_{VO_2} mm Hg	Flow ml/min/g	Arterial pressure mm Hg
Control	7.29 ± 0.02	47 ± 3	66 ± 7	2.5 ± 0.1	38 ± 4
Respiratory acidosis	6.86[b] ± 0.02	138 ± 3	109[b] ± 9	2.6 ± 0.1	47 ± 7
Metabolic acidosis	6.81[b] ± 0.03	42[c] ± 1	111[b] ± 17	2.5 ± 0.1	40 ± 5

[a] While coronary flow was maintained constant, perfusate was switched in random sequence to control solution, low HCO_3^- solution, or hypercapnic solution. pH_v, P_{vCO_2}, and P_{vO_2} refer to the coronary effluent.

[b] Significantly different from control.

[c] Significantly different from respiratory acidosis.

acidosis does indeed have a vasodilating effect upon the coronary vasculature. This vasodilating influence was masked in the present experiments, by an opposing influence exerted by the decrease in $M\dot{V}O_2$ secondary to acidosis. These results suggest that acidosis not only affects the coronary vessels directly, but also indirectly by changing the myocardial O_2 requirements. Accordingly, the net effect of a change of pH will depend on the relative influence of each of these two parameters. It is possible that failure to take into consideration the influence of changes in $M\dot{V}O_2$ during acidosis can account for the discrepant results obtained in the past.

The present results indicate that both respiratory and metabolic acidosis have a vasodilating effect on the coronary circulation. Moreover, when the same degree of acidosis was produced by either changing P_{CO_2} or HCO_3^- concentration similar quantitative changes in coronary vascular resistance were observed suggesting that one of the primary determinants of the changes observed is the pH of the perfusate.

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ERRATUM

Volume **147,** No. 1 (1974), in the article, "The Effect of Aortic Coarctation on the Concentrations of Water and Electrolytes in Cardiac Muscle," by P. Nachev, pp. 137–139:

Page 138, line 24 from top, should read: . . . potassium, and calcium concentrations (m*M*/liter) were . . . .