creas the acinar cells gave a strong reaction. In the liver the reaction appeared to be restricted to the parenchymal cells, and was more intense in cells adjacent to the central veins. The kidney gave no reaction. In the lungs the pseudostratified columnar epithelium lining the bronchi showed intense activity. In addition, other cells in the lung appeared to give a slight reaction; these may be the septal cells, since Gomori(4) states that septal cells of the adult lung contain esterase. The acinar cells of the thyroid gland gave a marked reaction both in the 15 and 20 mm stages of the mouse embryo and in the adult. Also active in the embryo were the pseudostratified columnar epithelium lining the nasal cavities and the so-called hibernating glands.

Discussion. The occurrence of esterase activity in the pancreas, liver, hibernating glands, and bronchial epithelium is in agreement with previous observations in adult mice and other mammals(4). Esterase activity has not previously been reported in the nasal mucosa; however, this activity might be expected, since the closely related bronchial epithelium contains the enzyme.

The esterase activity of both the embryonic and adult thyroid gland of the mouse is of particular interest since esterase has not been demonstrated in the thyroid glands of other animals previously studied. For example, Gomori(4) reports the absence of a histochemical reaction for esterase in the thyroid glands of man, monkey, dog, rabbit, guinea pig, and rat. Furthermore, Huggins and Moulton(7) who have made chemical studies of esterase activity in various organs of the rat and dog, report that the thyroid glands of these forms do not show a high level of esterase activity.

Quantitative microchemical methods are now being applied to embryonic organs to determine more precisely the rate of increase of esterase activity during development. In addition, because of the greater sensitivity of the chemical methods, esterase activity may be traced in the earlier embryonic stages which do not contain enough enzyme to give a positive histochemical test.

Summary. The 5 and 10 mm stages of mouse embryo do not show any histochemical evidence of esterase activity by the technic of Gomori. The distribution of esterase in the 15 and 20 mm stages of the mouse embryo, as demonstrated by the histochemical technic of Gomori, is essentially the same as that in the adult mouse. The only exception is the kidney, which does not give a positive staining reaction in the embryo. The occurrence of esterase activity in the thyroid gland seems to be peculiar to the mouse; the significance of this observation is not understood.

7. Huggins, C., and Moulton, S. H., *J. Exp. Med.*, 1948, v88, 169.

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Extension of Motile Life Span of Spermatozoa of the Domestic Fowl by Amino Acids and Proteins. (18973)

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Recent experiments(1,2) with sea urchins and other marine animals have shown that the addition of any one of a number of different amino acids and peptides to suspensions of the spermatozoa in sea water prolongs very greatly their functional life span. Both the

* Division of Poultry Husbandry, University of California, Davis. The experimental work was done at the Kerckhoff Laboratories of Biology while the senior author was on sabbatical leave at the California Institute of Technology.

1. Tyler, A., *Biol. Bull.*, 1950, v99, 324.

2. Tyler, A., and Atkinson, E., *Science*, 1950, v112, 783.

TABLE I. Influence of Glycine on Duration of Motility of Cock Sperm.

Glycine conc., molar	Control sol'n	No. of exp.	Semen conc., %	Max motility after 1st hr		Duration of motility		Relative life span (II/I)
				Control, %	Exp., %	I control, hr	II exp., hr.	
.033	⅓ s.w.	4	.3– .5	0	75– 90	<.1	6.5	>65
.033		4	.6– .7	0–75	75–100	<.1–4.5	>10.2–>11.1	2.3–>100
.033		3	1 –1.6	10–100	100	2.5–5.9	7–>11	1.4–>4.4
.067–.133		6	.6	10–75	25–100	2 –3	7.5–>11.1	2.5–>5.6
.167		2	.6	10–75	1	2;3	3.5;2.5	1.8;0.8
.013	Saline #1	1	.4	<1	5	1	8	8
.03		4	.3–.4	<1–1	25–100	1–3.5	8–10	2.9–10
.03		4	.6–1	1–10	50–100	2–3.5	7–>13	3.5–>4.5
.03	Saline #2	3	.3	10–75	50–100	4–7	8.5–13	1.8–2.1
.03		3	1 –1.2	25–50	75–90	7–14	9–>31	.6–2.4
.03		3	2.9–5	50–75	75–90	6.7–28	13.5–38	1.4–2

duration of motility and of fertilizing capacity are prolonged in presence of these agents. The present paper deals with the results of tests of these agents on cock sperm.

Material and methods. Semen was obtained by the method of abdominal massage(3) from single comb White Leghorn and Black Minorca cocks.† The semen samples were collected in glass tubes which were then placed in a thermos jug containing water at 15°C. The tubes were held above the water level so as to avoid too rapid cooling and possible temperature shock. The samples were kept in this condition for ½ to ¾ of an hour before dilution in the test solutions. The diluted sperm were incubated in a water bath at 22°C, using 10 ml samples in 50 ml glass stoppered Erlenmeyer flasks. In most experiments the flasks were shaken at 80 c.p.m. and 5 cm excursion. The percentage of motile sperm was estimated at regular intervals, microscopically, using semi-dark-field illumination. The total duration of motility or "life span" of the suspension is reported as the closest estimate of the time at which all the spermatozoa had become motionless.

A total of 53 tests were made with glycine at concentrations ranging from 0.013 to 0.167 M in salt solutions and with sperm densities ranging from 0.2 to 25% of semen. Two types of control diluents were used in different experiments: (a) sea water diluted 3-fold with distilled water to bring the osmotic pressure to a value tolerated by avian spermatozoa, and (b) 0.85% NaCl containing 0.214% $NaHCO_3$ and adjusted to pH 8.0 (saline No. 1). Ordinary distilled water and reagent-grade NaCl was used for saline No. 1. In certain later experiments glass redistilled water and Merck's NaCl for biological purposes was used for the preparation of the solution, and this is designated as saline No. 2. In the individual experiments the glycine solutions were prepared with the corresponding control diluent, using a stock glycine solution isotonic with the salt solution employed and at pH 8.0.

Results In Table I the results of 37 separate tests with glycine are summarized. That glycine may prolong the life span of cock spermatozoa is evident. In all of the experiments, with two exceptions to be discussed below, motility persisted longer in the solutions containing glycine than in the control solutions. The relative prolongation varied from 1.4 to more than 100 fold. A high degree of motility (*i.e.*, a high percentage of motile sperm) was also maintained better in these glycine solutions. This is illustrated in Table I by the maximum percentage motility observed after the first hour. There is, however, an upper limit to the effective concentration of glycine as demonstrated by the results

3. Burrows, W. H., and Quinn, J. P., *Poultry Science,* 1937, v16, 19.

† The animals used were from a flock at the George A. Richardson Breeding Ranch in San Gabriel, Calif. We are indebted to Mr. Marshall Richardson for placing these animals at our disposal and for his generous cooperation.

TABLE II. Effect of Concentrations of Glycine and of Semen on Duration of Motility of Cock Sperm. Experiment with a single pooled semen sample in Saline #2.

Glycine conc., molar	Semen conc., %	Max motility after 1st hr, %	Duration of motility, hr	Relative life span
0	.02	1	1	—
.03		5	7	7
.065		25	8	8
.1		50	6	6
.15		50	4	4
0	1	10	6	—
.03		10	8	1.3
.065		50	16	2.7
.1		75	8	1.3
.15		75	6	1
0	5	50	16	—
.03		90	24	1.5
.065		90	9	.6
.1		100	9	.6
.15		75	5	.3
0	25	90	32	—
.03		90	40	1.2
.065		90	50	1.6
.1		100	48	1.5
.15		90	50	1.6

with this amino acid at 0.167 M concentration. Glycine is isotonic with the control solution at this concentration and, consequently, was used without addition of any saline. In this solution life span was not prolonged and the degree of motility decreased rapidly, suggesting that either the high concentration or lack of salts may be toxic to the sperm. Significant prolongation was obtained with solutions ranging from 0.013 to 0.133 M (Table I). In an additional experiment, in which the total life span was not determined, sperm in solutions containing as little as 0.0033 M glycine were 50 to 75% active at least an hour after all sperm in the control solutions were dead.

As is well known, the life span of spermatozoa decreases with increasing dilution of the semen(4). This is evident in the present data on the control suspensions. The extent to which glycine prolongs the life span increases with decreasing concentration of semen, but not enough to overcome completely the effect of dilution (Table I). This tendency is comparable to that reported previously(1,2) for spermatozoa of invertebrate species.

Tyler and Rothschild(5) suggested that one way in which the amino acids might act in prolonging the life span of sperm is by binding certain highly toxic heavy metals ordinarily present in trace amounts in the diluents. On the basis of this consideration we prepared the saline No. 2, which contains considerably less of the heavy metals than the saline No. 1. In two direct comparisons of salines No. 1 and No. 2 using 0.3% semen, the life span was 1.2 hours in saline No. 1 and 4 and 5.5 hours in saline No. 2. With higher semen concentrations the life span in saline No. 2 was even more prolonged, and the relative prolongation due to glycine was correspondingly reduced. Glycine was still effective, however. In all but one of the trials listed in Table I the sperm lived longer in glycine than in saline No. 2.

A comparison of the relative effectiveness of semen concentration and glycine concentration on life span in saline No. 2 was obtained in a single experiment using one pooled sample of semen (Table II). The effect of semen concentration is clearly shown. Glycine appears to be only slightly effective in the higher sperm concentrations, but very considerably so in 0.2% semen. The optimum glycine concentration was 0.065 M, but, as in the results summarized in Table I, concentration was not sharply critical.

4. Rothschild, Lord, *Biol. Rev.*, 1951, v26, 1.

5. Tyler, A., and Rothschild, Lord, PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 52.

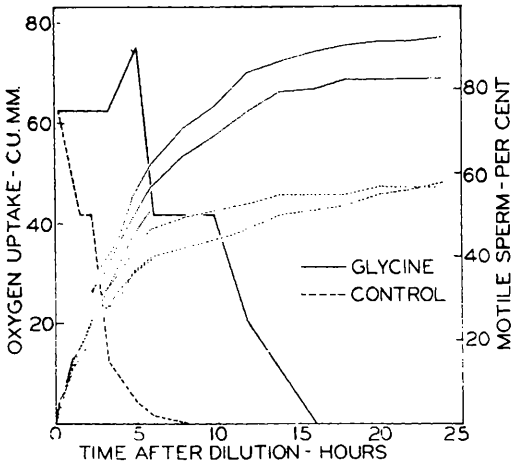

FIG. 1. Cumulative oxygen uptake and motility of senescing cock spermatozoa (5% semen suspension) in saline #2 with and without glycine (0.03 molar); (heavy lines denote sperm motility, light lines O_2 uptake).

Preliminary tests were also made of the effect of glycine on the oxygen uptake of cock sperm. The measurements were made in triplicate on 5% semen in conical Warburg flasks with saline No. 2 and with 0.03 M glycine in saline No. 2, each containing 50 units per ml of penicillin G. The antibiotic has proved useful(5) in inhibiting bacterial growth during prolonged respiration measurements on sperm suspensions. The degree of motility was followed on parallel samples in ordinary flasks and occasional samples from the respiration flasks. In this experiment the extension of motile life in the presence of glycine (Fig. 1) was much greater than that obtained in previous experiments (Table II) at this semen concentration. The total oxygen uptake in the presence of glycine considerably exceeds that of the controls, correlating roughly with the increased duration of motility. As in the experiments with sea urchin sperm(5) the presence of the amino acid enables these cells to maintain their oxidative metabolism longer than in its absence. Whether or not there are also significant initial differences in rate cannot be determined from the present measurements.

Diluted egg yolk and egg white are also effective in prolonging motility; 1% of the former and 2.5% of the latter in saline No. 1 produced prolongations approximately equivalent to optimum concentrations of glycine. Consequently, the effects of individual egg white proteins were studied. Of especial interest, in view of the hypothesis stated above, was the relative effectiveness of conalbumin, which binds iron and copper strongly(6,7), and the other components of egg white. For the same reason a derivative of an egg white protein, hydroxylamidoovomucoid, which is also a strong iron binder(6) was included in this study.‡ In saline No. 1 conalbumin prolonged the life span of sperm nearly twice as much as ovomucoid or as a preparation of egg white globulins (Table III). In saline No. 2 the relative effectiveness of these materials was the same, but the effects were only about half as great. Ovalbumin, which was studied only in saline No. 2, had no effect, but hydroxylamidoovomucoid more than doubled the life span in saline No. 2. In an additional experiment using saline No. 2 and 5% semen (not recorded in Table III) none of the proteins were effective, with the possible exception of hydroxylamidoovomucoid, which gave a relative life span of 1.3.

Discussion. Relatively few studies have been made of the life span of avian sperm *in vitro*. Grodzinski and Marchlewski(8) examined the effect of degree of dilution of the semen, temperature, pH, and certain diluents. The effectiveness of the diluents in maintaining motility was found to be, in decreasing order, avian blood serum, embryonic extract, egg white, and Tyrode's fluid. Later (9) they found that the avian sperm would survive in the blood serum of various vertebrates, but the results were complicated by the presence, in many of the sera, of natural

6. Fraenkel-Conrat, H., *Arch. Biochem.*, 1950, v28, 452.

7. Fraenkel-Conrat, H., and Feeney, R. E., *Arch. Biochem.*, 1950, v29, 101.

‡ The egg white proteins were prepared at the Western Regional Research Laboratory, Albany, Calif., and kindly supplied through the courtesy of Dr. R. E. Feeney.

8. Grodzinski, Z., and Marchlewski, J., *Bull. Acad. Polonaise des Sciences et des Lettres*, Cracovie, (Class des Sci. Math. et Naturelles), 1935, Series B, 347.

TABLE III. Influence of Proteins on Duration of Motility of Cock Sperm.

Material	Conc., %	Control sol'n	No. of exp.	Semen conc., %	Max motility after 1st hr		Duration of motility		Relative life span (II/I)
					Control, %	Exp., %	I control, hr	II exp. hr	
Conalbumen	.25	Saline	1	3	.4–.7	<1–10	25–100	1–3.5	9–11	3.1–10
	.1		1	1	.4	<1	10	1	6	6
	.25		2	1	1	25	25	7	26	3.7
Ovomucoid	.25		1	3	.4–.7	<1–10	1–50	1–3.5	3–4.5	1.3–3
	.1		1	1	.4	<1	<1	1	3.5	3.5
	.25		2	1	1	25	75	7	9.5	1.4
Globulins	.25		1	3	.4–.7	<1–10	5–10	1–3.5	4–6	1.7–4
	.1		1	1	.4	<1	1	1	4	4
	.25		2	1	1	25	25	7	12	1.7
Ovalbumin	.25		2	4	.3–2.9	25–75	25–90	7–<13	7–<13	.9–1.2
Hydroxylamido-ovomucoid	.25		2	3	.3–1.2	25–75	25–95	7–13	12–24	<1–3.4

heteroagglutinins for avian sperm. Various studies(10-13) have been made of the effect of storage temperature on fertilizing capacity of undiluted sperm, and the optimum is reported(13) to be between 10° and 20°C.

Munro(14) examined immediate effects of temperature and various diluents on motility without attempting to study its duration. He found that the motility of fowl sperm was lost at body temperature when suspended in physiological saline, Ringer's, Baker's, or Milanov's solution and in fluids expressed from the infundibulum and magnum, but he observed a high degree of motility in these diluents at lower temperature. Distilled water, seminal fluid, blood serum, uterine fluid, and egg white, on the other hand, supported motility at all temperatures studied (75-105°F). He(15) observed that the fertilizing capacity of sperm used immediately after dilution was reduced much more in Milanov's solution than in seminal fluid. By contrast with the above, Bonnier and Trulsson(16) observed no reduction in fertilizing capacity immediately after dilution with a modified Ringer's solution so long as the final semen concentration was at least 10%.

The results of the present investigation show that motile life span can be greatly extended simply by the addition of an amino acid to the suspending fluid as well as by addition of certain proteins. Whether or not fertilizing capacity is correspondingly extended, as in the experiments with sperm of invertebrates(1,2), has not as yet been determined. The present findings are also consistent with the suggestion(5) that the amino acids and proteins may act by virtue of their ability to bind certain heavy metals which are present in the usual salt solution diluents in trace amounts, and which may be highly toxic to the spermatozoa. Further studies of this and other possible interpretations of the limited duration of life span and fertilizing capacity of diluted sperm are contemplated.

Summary. Glycine, when added to saline diluents at concentrations between 0.003 and 0.133 molar, extends the life span of spermatozoa of the domestic fowl as measured by persistence of motility at 22°C. Glycine was relatively more effective on dilute than on concentrated semen suspensions. Certain

9. Grodzinski, Z., and Marchlewski, J., *Bull. Acad. Polonaise des Sciences et des Lettres,* Cracovie, (Class des Sci. Math. et Naturelles), 1938, Series B, 55.

10. Wheeler, N. C., and Andrews, F. N., *Poultry Sci.,* 1943, v22, 361.

11. Warren, D. C., and Gish, C. L., *Poultry Sci.,* 1943, v22, 108.

12. Burrows, W. H., and Quinn, J. P., Proc. Seventh World's Poultry Congress, 1939, 82.

13. Garren, H. W., and Shaffner, C. S., *Poultry Sci.,* 1950, v29, 758.

14. Munro, S. S., *Quart. J. Exp. Biol.,* 1938, v27, 281.

15. Munro, S. S., *Canadian J. Research, D,* 1938, v16, 281.

16. Bonnier, G., and Trulsson, Sally, Proc. Seventh World's Poultry Congress, 1939, 76.

egg-white proteins had a similar effect on the life span of sperm. Conalbumin and a derived protein, hydroxylamidoovomucoid, were more effective than other proteins from egg white. Purification of the saline diluent alone served to prolong the life span of spermatozoa and correspondingly reduce the effects of glycine and proteins.

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Production of Cirrhosis and Liver Tumors in Male Rats Using High Dosages of 2-Acetylaminofluorene.*† (18974)

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Since Wilson, De Eds, and Cox, Jr.(1) introduced 2-acetylaminofluorene (2-AAF) as a new experimental carcinogen, the compound has been widely used, due to its powerful carcinogenic action and the unusual variety of benign and malignant tumors produced in different organs. 2-AAF appears to be of particular value in studies of the mode of development of cirrhosis and liver tumors. The possibility of correlation of liver changes with lesions produced in other locations seems to confer on 2-AAF an advantage over compounds with strictly hepatotoxic action.

Methods. One hundred and forty-eight rats of hooded strain were used, comprising 73 males and 75 females. The rats were derived from a local strain, inbred since 1933 by continuous brother and sister mating. Two animals of the same sex were kept in each cage. The diet was fully adequate, consisting of a standard Purina meal preparation and water *ad libitum*. The 2-AAF was added to the diet (by mixing) for 35 consecutive weeks. The age of the animals at the start of the experiment was 4 weeks in 128 rats (53 males and 75 females) (Group I), and 16 weeks in 20 males (Group II). The absolute dose of the 2-AAF was 4 mg per day in Group I and 7.5-9 mg per day in Group II for the first 7 weeks; afterwards it was lowered to 3 mg and 6 mg per day respectively for the remaining period of time.

It soon became apparent that male rats receiving higher absolute daily dosages of 2-AAF (Group II) developed extensive liver changes in a short period of time. Twelve of them died within 39 days of the start of administration of the compound. Partial hepatectomies were performed at intervals of 4 to 5 weeks for 7 months on the remaining rats of this group, starting from the third month. Under light ether anesthesia and using sterile technic, 20-25% of the liver volume was removed each time through a mid-line abdominal incision. Although the total amount of liver tissue resected by repeated hepatectomies exceeded the original liver volume, the majority of the animals survived all operations. Control hepatectomies were done on 6 animals kept on the lower absolute daily dosage of 2-AAF (Group I). Hematoxylin-and-eosin and connective-tissue-stain sections were prepared from the resected portions of the liver.

Results. Pathological findings observed by repeated hepatectomies. The earliest gross liver changes in the older group of male rats were observed during the second month by examining the 11 rats which died in this period. The liver was decreased in size with multiple white nodules protruding on the surface. Microscopically, the nodules consisted of circumscribed areas of hyperplasia without specific relation to lobular pattern (Fig. 1).

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† This work was presented at Clinical Congress of American College of Surgeons, Boston, Mass., Oct. 1950. Abstract in *Trans. Surg. Forum*, 1950, v1, 205.

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1. Wilson, R. H., De Eds, F., and Cox, A. J., Jr., *Cancer Res.*, 1941, v1, 595.