tion of histamine should also stimulate secretion by the oxyntic cells. However, in the presence of damage acid secretion is difficult to detect, for the plasma-like fluid buffers and dilutes hydrogen ions, and increased mucosal permeability allows hydrogen ions to disappear by diffusing back into it(10).

Summary. The stomachs of anesthetized, pylorus-ligated rats were filled with 154 mM NaCl, 100 mM HCl, 100 mM acetic acid or 20 mM salicylic acid in 100 mM HCl. After 90 min the fluids were removed, and they as well as the oxyntic gland area of the stomachs were analyzed for histamine. There was a rise in the frequency with which histamine could be detected in the acetic and salicylic acid solutions as compared with NaCl or HCl, and its mean concentration also increased. Gastric tissue exposed to acetic acid or salicylic acid contained about two-thirds as much histamine as did tissue exposed to NaCl or HCl. We infer that during damage histamine is present in the mucosal interstitial fluid where it can exert its characteristic physiological effects.

We thank Joseph Kennedy of the Section of Physiology, Mayo Clinic, for teaching us the histamine assay.

1. Davenport, H. W., Gastroenterology, 1964, v46, 245.
2. ———, ibid., 1965, v49, 189.
3. ———, ibid., 1965, v49, 238.
4. Foley, W. A., Glick, D., ibid., 1962, v43, 425.
5. Clark, B. B., Adams, W. L., ibid., 1947, v9, 461.
6. Räsänen, T., ibid., 1961, v40, 232.
7. Code, C. F., McIntire, F. C., in Methods of Biochemical Analysis, Glick, D., Ed., Interscience, New York, 1956, v3, 49.
8. Barsoum, G. S., Gaddum, J. H., J. Physiol., London, 1935, v85, 1.
9. Code, C. F., Hallenbeck, G. A., Gregory, R. A., Am. J. Physiol., 1947, v151, 593.
10. Davenport, H. W., Warner, H. A., Code, C. F., Gastroenterology, 1964, v47, 142.

Received September 9, 1965. P.S.E.B.M., 1966, v121.

A Liver Glycogen Assay of Anti-Inflammatory Steroids on Chicks and Embryos. (30785)

JOHN NEWMAN* AND LASZLO GYERMEK
Syntex Institute of Hormone Biology, Syntex Research Center, Palo Alto, Calif.

There is a continuous interest in increasing sensitivity and accuracy of hormonal bioassays. Detection of trace amounts of hormones utilizing biological tests may offer advantages over certain chemical determinations, which are many times non-specific, and may yield misleading results by measuring chemically similar but biologically different metabolic products. Therefore, a sensitive assay for corticosteroids and synthetic anti-inflammatory steroids was sought. The present study is based on earlier reports on the high sensitivity of chicken embryos to natural corticosteroids, as represented by suppression of growth(1,2), and on a more recent study of Guzman-Garcia *et al*(3) including observations on water, electrolyte, and carbohydrate metabolism of the developing chick embryo as influenced by synthetic corticosteroids. The latter publication indicated that some of the fluorinated anti-inflammatory steroids are extremely potent in influencing growth as well as liver glycogen content, and suggested that by further analysis of the liver glycogen, selecting optimal dose and time intervals, a sensitive bio-assay procedure can be developed.

Methods. (a) Fertilized white Leghorn eggs were obtained at 9 to 10 days of incubation (Kimber Farms, Inc., Fremont, Calif.) and were inoculated at the 9th, 12th, 13th, or 16th day of incubation into the yolk sac. Steroids were dissolved in sterile corn oil and were injected in a volume of 0.05 ml. The embryos were sacrificed 1-7 days after the injections. Livers were dissected free, and

* Present address: Virus Laboratory, University of California, Berkeley.

TABLE I. TCA-Extractable Liver Glycogen Content of Chick Embryos at Different Stages of Development. Effect of Cortisone and Fluocinolone Acetonide.

Days of development	Liver glycogen content: mg/100 g (± S.D.)					
	Controls	Oil injected controls	Cortisone 10 μg	Cortisone 50 μg	Fluocinolone acetonide .01 μg	Fluocinolone acetonide .05 μg
12	.84 (.14)					
14	.95 (.42)	.92	1.77 (.66)	3.59*(.80)	1.92 (.61)	2.18 (.76)
15	1.33*(.47)	1.84*(.81)				6.24 (.50)
16	1.22 (.45)	1.03*(.49)	1.68 (.26)	2.05 (1.6)	1.99*(1.07)	5.49*(.47)
19	1.89 (.56)	1.94 (.32)	1.69 (.13)	2.28 (.52)	2.38 (.37)	5.04 (1.9)

Values listed were averages of 4-5 samples.
Values with * indicate average of 8-9 samples.

about 200 mg samples were immersed in 1.5 ml 10% trichloracetic acid (TCA) and homogenized immediately. The weight was determined after homogenization, reweighing the tubes and contents. The TCA extractable glycogen content was determined according to the method of Gyermek and Fekete (4), with the modification that instead of filtering the TCA-liver precipitate, it was centrifuged. One ml of supernatant was removed and 1 ml of absolute alcohol added, followed by thorough mixing, then allowed to stand for 15 minutes at room temperature. The precipitate was kept in suspension by mixing, transferred to a cuvette, and optical densities read on a Leitz Model M photometer using the yellow filter (580 mμ). In the preliminary experiments 3-5, in the bio-assay experiments, 4 to 14 liver samples were used at each dose level. A standard glycogen calibration curve was obtained by using a glycogen sample prepared from chicken livers.

(b) One-day-old fasting Leghorn chicks were given 2 anti-inflammatory steroids dissolved in oil orally (total volume 0.05 ml) through a syringe. The animals were sacrificed 17 hours later and the liver glycogen determined as described above.

Results. 1. TCA-extractable glycogen content of untreated chick embryos. This liver glycogen fraction represents a major portion of the total and undergoes changes with the maturation of the embryo similar to those observed with the total glycogen content. These changes are represented in Table I. There is no marked increase until the 16th day of incubation. At the 19th day, the values are about 2 times higher than at 12 days. Similar data were reported by Guzman-Garcia *et al*(3), who found moderate increase of total liver glycogen between the 12th and 14th day and a marked increase at 18 days of incubation.

2. Onset and duration of glycogenic action of cortisone as compared to fluocinolone acetonide. Embryos were injected on the 12th day of incubation, and liver glycogen determined at 14, 15, 16, and 19 days. The results are shown in Table I, indicating that: (a) Oil controls (.05 ml of corn oil) showed a slight increase of liver glycogen content at 14 days as compared to the uninjected controls. (b) The response to cortisone was at its maximum on day 14 and declined by day 16. (c) In contrast to cortisone, the effect of fluocinolone was slower developing. Maximum response was obtained at days 15 and 16, and only moderate decrease in the glycogen content occurred at day 19, indicating a considerably slower degree of inactivation of the latter steroid.

3. Influence of anti-inflammatory steroids on the liver glycogen content on embryos treated at 12 days of age. (a) Fluocinolone acetonide: (6 alpha, 9 alpha, difluoro pregna-1,4-diene, 11 beta, 16 alpha, 17 alpha, 21 pentol, 16, 17 acetonide (Synalar®). The influence of this agent on the liver glycogen of the developing chick embryos has been analyzed in detail. Doses as low as .012 μg injected at 12 days of incubation produced significant increase in the liver glycogen content at the 15th and 16th day (Table II). A series on embryos of 9 days of incubation (Table II) indicated a somewhat higher degree of sensitivity but a lower degree of accuracy of the assay. Considering also the small size of the liver of these embryos, it

TABLE II. Changes of Liver Glycogen Produced by Fluocinolone Acetonide in Chick Embryos of Different Age.

Age when injected	Age when analyzed	Dose μg	Liver glycogen (± S.D.) (g/100 g tissue)	n	Slope	λ
9	14	—	.59 (.27)	5	2.86	.23
		.006	1.22 (.27)	5		
		.012	2.30 (1.06)	5		
		.025	2.94 (.63)	5		
12	16	—	1.25 (.33)	7	4.83	.11
		.006	1.14 (.43)	9		
		.012	1.99 (.75)	8		
		.025	2.97 (.43)	6		
		.05	5.50 (.49)	7		
16	20	—	1.73	3		
		.025	1.50	3		
		.10	4.3	3		

n = number of samples. λ = index of precision.

was not practical to use eggs at the earlier developmental stage, then 12 days. Sensitivity at a later state of development was also tested at 16 days of incubation, sacrificing the embryos at 20 days. The doses necessary to produce measurable increase of liver glycogen were 2 to 3 times larger than at 12 days (Table II). Since the weight of the embryo at 16 days is also about 2-3 times the weight of that of a 12-day-old, the difference in sensitivity is due rather to the increased body weight than to the change of inactivation rate of the steroids in the older embryo. (b) Other anti-inflammatory steroids: Cortisone and 3 other synthetic anti-inflammatory steroids: flurandrenalone (acetonide)- (6α-fluoro, 16α hydroxyhydrocortisone acetonide), flumethasone- (6α,9α difluoro, 16α methyl, 11β, 17α, 21-trihydroxypregna-1, 4-dione and prednisolone were tested for their glycogenic activity. The embryos were treated on the 12th day of incubation and were sacrificed on the 16th day, except for cortisone, which was assayed on the 14th day. The results are presented in Table III.

The extremely low dose levels in Tables II and III indicate that the chick liver glycogen test is a very sensitive assay procedure for anti-inflammatory steroids. For example, the threshold dose of fluocinolone acetonide producing significant change in liver glycogen was .012 μg. The sensitivity of the most commonly used bio-assay procedures, *i.e.*, thymus atrophy test, is about 50 times lower than that.

The accuracy of the embryo assay is satisfactory with the highly potent anti-inflammatory steroids studied. The λ values varied between .11 and .33. A decreasing degree of accuracy seems to exist, however, with the decrease in potency of the steroid. This might be due to a toxic effect of the larger quantities of the less potent compounds (cortisone, prednisolone) which may interfere with the linear increase of the liver glycogen at the higher dose levels, thereby suppressing the slopes. Such an effect was indicated by the fact that the 2 lowest slopes were found with the 2 least potent compounds.

(c) Bio-assay of anti-inflammatory steroids on the 1-day-old chicks. Two anti-inflammatory steroids (fluocinolone acetonide and flumethasone) were tested on 1-day-old chicks as mentioned in *Methods*. Results are presented in Table IV. Although a definite dose response correlation can be detected, the precision and sensitivity of this assay utilizing oral administration of steroids is inferior to that of the embryo assay. The oral administration, however, offers certain advantages; for example, in detecting residues of anti-inflammatory steroids in animal tissues. The glycogen assay, even in this less sensitive form, still seems to be of more value than other less specific, less sensitive and probably also less accurate procedures of detecting anti-inflammatory steroids by continued feeding into newborn chicks measuring the growth curve of the animals. An additional advantage of the glycogen assay

TABLE III. Effect of Some Anti-Inflammatory Steroids on Liver Glycogen of Chick Embryos.

Compound	Dose in μg	Liver glycogen (± S.D.) (g/100 g tissue)	n	Slope	λ
Cortisone	—	1.02 (.27)	8	1.78	.38
	10	1.38 (.40)	8		
	20	2.15 (.79)	9		
	40	3.18 (.89)	7		
	80	2.98 (.62)	8		
Prednisolone	—	1.05 (.25)	9	1.88	.58
	.5	1.04 (.49)	11		
	1.0	1.38 (.79)	11		
	2.0	1.95 (1.20)	12		
	4.0	2.1 (1.22)	8		
	8.0	3.3 (1.74)	4		
Flurandrenolone	—	1.23 (.24)	6	3.34	.33
	.025	1.98 (.54)	7		
	.05	2.23 (.77)	10		
	.1	4.60 (1.54)	8		
	.2	5.00 (1.63)	8		
Flumethasone	—	.96 (.35)	10	6.77	.18
	.025	.88 (.25)	11		
	.05	2.22 (1.18)	14		
	.1	4.89 (2.21)	11		
	.2	6.99 (1.26)	8		

n = No. of samples.
Embryos were injected at 12 days and assayed at 16 days.

TABLE IV. Effect of Fluocinolone Acetonide and Flumethasone on Liver Glycogen of 1-Day-Old Chicks.

Compound	Dose in μg	Liver glycogen (± S.D.) (g/100 g tissue)	n	Slope	λ
	—	1.44 (.15)	9		
Fluocinolone acetonide	1.0	1.41 (.20)	7	2.08	.27
	2.0	1.98 (.34)	9		
	4.0	2.43 (.74)	8		
	8.0	3.29 (.95)	8		
Flumethasone	—	1.46 (.32)	11	1.29	.34
	.5	1.62 (.37)	11		
	1.0	2.06 (.54)	11		
	2.0	2.63 (.38)	11		
	4.0	2.78 (.47)	11		

is its relative rapidity. The results are obtained within 24 hours in the case of the newly hatched chicks and within 4 days in the case of using embryos. Analysis of variance of the data as obtained with fluocinolone acetonide in the embryo and 1-day-old chick experiments was calculated and variance ratios$_{(F)}$ of 8.79 for the chick embryo series and of 14.64 for the 1-day-old chick series were obtained. Analysis of curvature of the dose-response regression lines indicated that they did not significantly differ from straight lines ($P=>.5$).

Summary. A bio-assay procedure for highly potent anti-inflammatory steroids is described utilizing the changes in TCA-extractable liver glycogen content of either chick embryos at 12-16 days of incubation or of newly hatched chicks. The embryo assay is more sensitive than other commonly used bio-assay procedures for determination of anti-inflammatory steroids. Newly hatched chicks react to lesser extent, but seem to be suitable for rapid detection of small amounts of anti-inflammatory steroids in crude biological samples (*i.e.*, residues in animal tissues).

The authors express their thanks to Mr. James Work for statistical evaluation of the data.

1. Sames, G. L., Leathem, J. H., Proc. Soc. Exp.

Biol. and Med., 1951, v78, 231.
2. Karnofsky, D. A., Ridgeway, L. D., Patterson, P. A., Endocrinology, 1951, v48, 596.
3. Guzman-Garcia, J., Gomez-Puyou, A., Pena-Diaz, A., Laguna, J., Steroids, 1963, v1, 423.
4. Gyermek, L., Fekete, G. Y., Acta Physiol. Acad. Sci. Hung., 1955, v8, p259; Nature, 1955, v175, 386.

Received September 9, 1965. P.S.E.B.M., 1966, v121.

Ficoll Activation of a Protein Essential for Maturation of the Free-Living Nematode *Caenorhabditis briggsae.** (30786)

EDWARD J. BUECHER, JR., EDER HANSEN AND EVANGELINE A. YARWOOD
Kaiser Foundation Research Institute, Richmond, Calif.

Previous work with axenic culture of the free living nematode, *Caenorhabditis briggsae,* led to isolation of a heat labile fraction from liver which was essential for maintenance of these cultures in an otherwise chemically defined medium(1,2). Subsequently, this fraction has been further separated by chromatography on hydroxylapatite(1A). The globulin recovered has the interesting property of being "activated," that is, certain specific treatments increased the biological activity 30-fold. This phenomenon may have a bearing on the availability of proteinaceous materials in biological systems. This proteinaceous growth factor is present in most animal tissues as well as in bacteria. Preliminary studies show that the material is effective in a wide range of biological systems.

The first method of activation discovered was freezing the growth factor in the basal defined medium. This activation proved to be limited in stability and restricted to relatively acid media. The need to expand the cultural conditions led to reexamination of the activation process and to the development of other methods of producing this effect. Recently activation has been produced by dialysis of growth factor to low phosphate, by controlled periods of heating, by lowering the pH of the defined medium to 5.6, and by addition of proper levels of the sucrose polymer Ficoll (Pharmacia).

With several methods available for activation, the next step was a critical evaluation of the effects of each separately and in combination with each other. In this the outstanding results obtainable with Ficoll soon became evident and increased emphasis was given to this phase of the investigation.

Materials and methods. The free-living, self-fertilizing protandrous nematode *C. briggsae* was used under axenic conditions in all assays. Stock cultures were maintained in a medium containing liver extracts(2,3). Larvae used in assays were derived from eggs laid by young adults(1). Three larvae were inoculated into duplicate 10 × 75 mm tubes containing 0.25 ml aliquots of the medium to be tested.

Effectiveness of culture media was measured by time for maturation and reproduction(4,1). Time of maturation was determined by observations at ½ day intervals and calculated to 0.1 day based on the rate of appearance of F_1 larvae. Previous observations had shown that the rate of larvae produced at the 100 μg/ml level averaged one larva per adult every 2½ hours, *i.e.,* 0.1 day. The time at which the first larva hatched was thus calculated to 0.1 day. Worms were observed for 20 days, any worm not producing an F_1 by this time being classified as a non-maturer. Degrees of effectiveness of different media were compared by rates of maturation expressed as relative response. An F_1 time of 2.8 days, obtained when worms were grown with *E. coli* on agar slants(1), was considered the maximum response (=100).

The medium used to measure dose response consisted of two components(1), the basal medium of chemically defined ingredients and the proteinaceous growth factor preparation. The growth factor used for these experi-

* Supported in part by U.S.P.H.S. Grants AI 00926 and AM 06004.