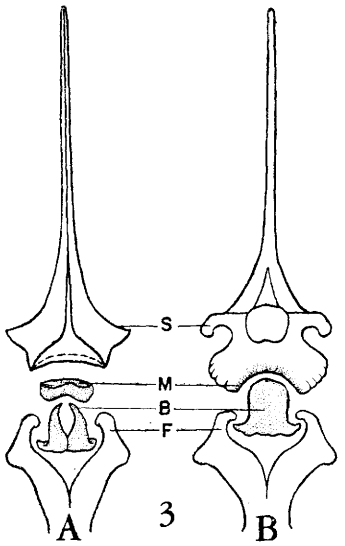

FIG. 3. Anterior view of a unit of the anal fin suspensorium at the level of the fifth interhemal spine (S). A. Normal adult female: the mesonost (M) and baseost (B) are discrete, the baseost being bipartite. B. Normal adult male: the mesonost (M) is fused to the base of the interhemal spine (S) and the baseost (B) is unipartite. Abbreviations: S—interhemal spine; M—mesonost; B—baseost; F—anal fin ray.

comprise the basal portion of the anal fin suspensorium. In normal adult females they are discrete; the baseosts being bipartite (Fig. 3A). In normal adult males, the mesonosts are apparently missing, but they are actually fused to the ventral surfaces of the interhemal spines (Fig. 3B). The baseosts in males are discrete but unipartite.

In 6 of the 12 adult females treated with methyl testosterone for 8 weeks, the entire series of mesonosts was fused to the ventral surfaces of the first 8 interhemal spines. In the 6 remaining animals, the anterior mesonosts were fused while the posterior ones were close to but not fused to their corresponding interhemal spines. In all of the treated animals, the baseosts had begun to take on the unipartite condition characteristic of normal males. The 7 control females had normal, discrete mesonosts and bipartite baseosts.

Summary. Alpha-estradiol benzoate induced the dissolution of one or two anterior hemal spines in a significant number of immature and maturing male platyfish but elicited no response in adult males. Methyl testosterone, administered to adult female platyfish, induced the fusion of 2 series of small, basal bony elements, the mesonosts and baseosts. The mesonosts ordinarily discrete, became fused to the ventral surfaces of the interhemal spines. The baseosts, normally bipartite, became unipartite.

1. Hisaw, F. L., *J. Exp. Zool.,* 1925, v42, 411.
2. Gardner, W. U., *Am. J. Anat.,* 1936, v59, 459.
3. Vivien, J., and Mohsen, T., *Compt. Rend. Soc. Biol.,* 1951, v145, 1364.
4. Rubin, A. A., Thesis, 1953, Library, New York University.
5. Gordon, M., *Genetics,* 1927, v12, 253.
6. ———, *Genetics,* 1947, v32, 8.
7. Grobstein, C., *Univ. Calif. Pub. Zool.,* 1940, v47, 1.

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Rate of Disappearance and Metabolism of Hydrocortisone and Cortisone in the Synovial Cavity in Rheumatoid Arthritis.*† (20447)

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Several investigators have found that the acetate of hydrocortisone (Kendall's Compound F, 11-hydroxy-corticosterone) is effective in suppressing inflammation when injected into joints with synovial effusions(1-3). Since

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the synovial fluid is easily accessible to analysis, this observation affords an opportunity of studying some of the processes which may accompany the therapeutic response in the arthritic joint.

Cortisone acetate has been found to be far less active than hydrocortisone acetate when administered intra-articularly, while both are known to relieve rheumatoid arthritis when administered systemically. It was of interest, therefore, to determine whether difference in their rates of absorption from the synovial cavity would account for the difference in activity. Accordingly quantitative assays have been performed on extracts of synovial fluid aspirated at various time intervals after the injection of cortisone, hydrocortisone and their acetates.

Another explanation for the difference in local effectiveness might be that cortisone must be converted to hydrocortisone in order to become therapeutically active, and that this transformation cannot adequately be effected by synovial tissue, though it does presumably occur elsewhere in the body when cortisone is given systemically. It is also possible, finally, that both compounds must be converted to an active substance, as yet unknown, before exerting their effect at the site of inflammation. A search for metabolites of cortisone, hydrocortisone and their acetates in the synovial fluid was, therefore, undertaken.

Experimental. Injection of compounds and aspiration of fluid. The 6 subjects were patients with rheumatoid arthritis who had effusions in the knee joints. They were receiving maintenance cortisone therapy in a dosage of 75 to 100 mg of cortisone acetate per day orally. In most experiments a control sample of synovial fluid was withdrawn before injection of steroid. If a subject had effusions in both knees, the entire fluid from one knee was used as control. If only one effusion was present, approximately half the fluid was withdrawn for control analyses before injection of steroids. In several instances a 2-week interval was allowed after aspiration of all the fluid for control analyses, during which the fluid reaccumulated, and the injections were then performed.

Cortisone, hydrocortisone, and their acetates, in quantities of 25 to 100 mg were injected into the knees in saline suspension.‡ The knees were flexed and massaged repeatedly to effect mixing in the synovial fluid, and after various time intervals all the fluid was withdrawn. Two 20 ml portions of physiological saline solution were then injected, and withdrawn after further flexion and massage. After noting the volumes, the original fluid was combined with the saline washes. In a number of experiments it was possible repeatedly to recover 20 ml volumes of injected physiological saline completely, and it is believed, therefore, that the synovial fluid containing the injected compounds as well as the saline washes were also accessible to complete removal.

Model experiments were performed to test whether simple settling out to an inaccessible region of the synovial cavity would be an appreciable factor in recovery experiments. Twenty-five ml of synovial fluid aspirated from the knee joint of a patient with rheumatoid arthritis was placed in a cellophane dialyzing bag. One ml (25 mg) of cortisone acetate suspension was pipetted into the center of this fluid. The suspended steroid immediately rose to the top of the fluid. The bag was tied off just above the upper fluid level, then alternately squeezed, released, and bent, in simulation of flexing and massage of the knee joint. After one hour, approximately 5 to 10% of the steroid material had settled to the bottom, leaving most of the steroid suspended.

Extraction. The combined synovial fluid and saline washes were diluted with nine volumes of distilled water, shaken to break up the viscous masses of mucoid material, then extracted three times in separatory funnels, with a volume of chloroform equal to one-third the volume of the diluted fluid. After each extraction the resulting emulsion was centrifuged, and the aqueous layer obtained was recombined with the main portion. The combined chloroform extracts were washed once with cold 5% sodium carbonate solution and 3 times with distilled water. The chloroform

‡ This was the saline diluent provided by Merck and Co. which contains suspending agents and 0.9% benzyl alcohol.

TABLE I. Recoveries of Cortisone, Hydrocortisone, and Their Acetates from Synovial Fluid and Water.*

Procedure controls

Exp. No.	Compound added	mg added	Vol extracted, ml: Water	Vol extracted, ml: Synovial fluid	% recovered by assays†: FS	% recovered by assays†: MRS	% recovered by assays†: PS‡
1	E free	25.0	250	0	81	75	86
2	"	.5	250	0	86	70	67
3	F ac	25.0	225	25	§	—	68
4	"	"	"	"	§	—	63
5	E free	"	"	"	86	68	63
6	F free	"	"	"	93	64	65
				Avg	89	66	65
7	E ac	.5	225	25	§	—	54
8	F ac	"	"	"	§	—	60
9	E free	"	"	"	106	133	90
10	F free	"	"	"	68	52	27

* E is cortisone; F is hydrocortisone; E ac and F ac are their acetates.

† FS, formaldehydogenic steroids; MRS, molybdate reducing steroids; PS, Porter-Silber reacting steroids.

‡ Calculated in equivalents of the compound listed as a reference standard.

§ The acetates do not react in the formaldehydogenic assay.

was then evaporated to dryness at temperatures not over 40°C, *in vacuo,* and the residue quantitatively transferred to a small vial.

Quantitative assays. 1) Porter-Silber. All extracts were assayed by a micro modification of the Porter-Silber reaction(4) using phenylhydrazine in sulfuric acid. The appropriate standard was used if the compound to be assayed was known. Unknown mixtures are expressed as mg cortisone equivalent.

2) Periodic acid oxidation. The formaldehyde liberated by periodic acid was determined by a micro modification of the direct reaction of Lowenstein, Corcoran and Page (5).

3) Molybdate reduction. A micro adaptation of the acid molybdate reduction assay of Heard and Sobel(6) was employed for some extracts.

Paper chromatography. The procedure of Burton and Zaffaroni(7,8) was employed without major modifications. The steroids were located by cutting 2 mm wide test strips from the main chromatogram, and applying in turn the triphenyl tetrazolium chloride reduction test(7), the osmium tetraoxide reduction test (9), and by noting the areas of fluorescence after application of concentrated sulfuric acid (8). The chromatograms were developed in toluene-propylene glycol (diluted 1:1 with methanol) for periods varying from 5 to 9 days, in order to separate slow moving compounds, after which the runoffs were reapplied and developed for shorter periods in order to yield the faster moving compounds.

Results. 1. *Procedure controls.* Control recovery experiments were performed in order to check the precision of the method. Table I shows that when 25 mg of cortisone alcohol were added to 250 ml of water, and put through the entire extraction and assay procedure, an average of 81% was recovered by the 3 assay methods. When the 4 steroids were added to 25 ml of human synovial fluid, which was then diluted with 225 ml of water, the recoveries were somewhat lower by the reduction and Porter-Silber methods. When 0.5 mg quantities were added, the values showed greater deviations from the mean assay.

2) *Post injection recovery experiments.* Table II summarizes the assay findings on synovial fluids withdrawn at various intervals after injection of cortisone and hydrocortisone acetates and free alcohols. Values in the 3 right hand columns have been corrected for the percentage recoveries obtained in the procedure control experiments. When the fluid was removed only 5 minutes after injection of 50 mg of steroid, there was already an average diminution of about 40% in the corrected assay. One hour after the injection of

TABLE II. Recoveries of Hydrocortisone, Cortisone, and Their Acetates* after Injection into Synovial Cavity.

Compound injected	Patient	mg injected	Interval before aspiration (min.)	% recovery by assays† Uncorrected FS	MRS	PS	Corrected for procedure controls‡ FS	MRS	PS§
E ac	G. R.	50	5	—	—	30	—	—	46
F free	G. R.	50	5	35	—	56	39	—	86
F ac	E. S.	50	60	—	—	6	—	—	9
"	M. F.	50	60	—	—	9	—	—	14
"	G. R.	25	60	—	—	14	—	—	21
E ac	G. R.	50	60	—	—	8	—	—	12
"	H. G.	100	120	—	—	13	—	—	20
"	R. M.	50	480	—	—	<1	—	—	<1
F free	G. R.	100	30	16.1	14.2	18.3	18.1	21.5	28.2
"	G. R.	100	30	18.0	18.9	10.5	20.2	28.7	16.2
"	G. R.	100	30	7.9	9.9	10.2	8.9	15.0	15.7
"	E. W.	100	30	21.3	16.5	18.1	23.9	25.0	27.8
			Avg	15.8	14.9	14.3	17.8	22.6	22.0
F free	N. B.	100	180	1.1	2.7	5.0	1.2	4.0	7.7
"	G. R.	100	180	.3	1.4	1.1	.3	2.1	1.7
"	G. R.	100	180	.6	1.1	1.2	.7	1.7	1.8
"	G. R.	100	180	.3	.8	.4	.3	1.2	.6
			Avg	.6	1.5	1.9	.6	2.3	3.0
E free	L. M.	100	180	3.4	7.1	1.6	3.8	10.7	2.5
"	G. R.	100	180	2.5	4.6	1.2	2.8	7.0	1.8
"	G. R.	100	180	.8	5.0	.5	.9	7.6	.8
"	G. R.	100	180	.8	3.4	1.0	.9	5.1	5.2
			Avg	1.9	5.5	1.1	2.1	7.6	2.6

* E is cortisone; F is hydrocortisone; E ac and F ac are their acetates.

† FS, formaldehydogenic steroids; MRS, molybdate reducing steroids; PS, Porter-Silber reacting steroids.

‡ Each assay is corrected for the avg % recovery of exp. 3, 4, 5 and 6 shown in Table I.

§ Calculated in equivalents of the injected compound as a reference standard.

hydrocortisone acetate and cortisone acetate the steroid remaining had decreased to an average of 14% of that injected. In four identical experiments 30 minutes after the injection of 100 mg hydrocortisone free alcohol, the average recovery was 21%. Similar experiments 3 hours after injecting 100 mg of either hydrocortisone or cortisone free alcohols gave an average recovery of only about 2%.

3. *Paper chromatographic analyses.* A series of extracts prepared from synovial fluid samples of a single subject (G. R.), after the injection of hydrocortisone free alcohol, were submitted to chromatographic analysis on paper. On each occasion 50 mg of the steroid were injected, and the entire synovial fluid subsequently withdrawn and combined with saline washings. The results are depicted in Fig. 1. Porter-Silber assays on the crude extracts showed that 29.2 mg or only 56% of the injected steroid could be recovered from the synovial fluid 5 minutes after injection. In the half-hour and one hour experiments the assays were 4.4 mg and 0 mg respectively, again showing the rapid disappearance of the injected steroid.

The left hand chromatogram in Fig. 1 shows the approximate position of known pure compounds run on parallel strips when 100 μg were applied on a one cm paper and tested by the reduction spot tests. The second chromatogram of Fig. 1 shows the results with the combined pre-injection control fluid extracts, run on one cm papers. Since this patient had been receiving 100 mg of cortisone acetate orally per day for a period of 18 months, it is interesting to note that no cortisone (compound E) could be seen on the control fluid chromatogram. Only one faint band was seen, indicating the presence of about 50 μg of a substance having a mobility similar to that of hydrocortisone (compound F). The third chro-

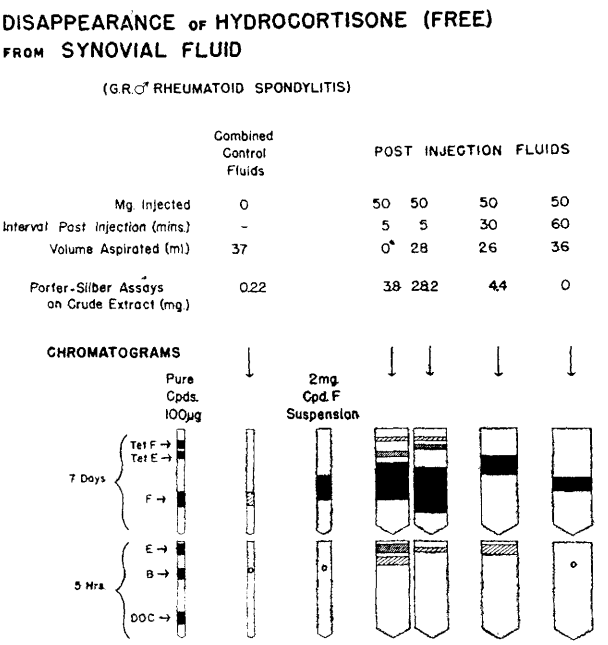

FIG. 1. Disappearance of hydrocortisone (free) from synovial fluid. "Tet F" is tetrahydrohydrocortisone; "Tet E" is tetrahydrocortisone; "F" is hydrocortisone; "E" is cortisone; "B" is corticosterone; "DOC" is 11-desoxycorticosterone. Although tetrahydro-hydrocortisone was not available as a pure reference standard, its mobility is believed to be as shown on the chart, by analogy both with isomeric adrenal cortical compounds, and with chromatographic fractions of urine extract shown to contain this compound.

* No effusion was present in the joint when this experiment was done. The steroid recovered was obtained by washing the joint with two 20 ml portions of saline solution.

matogram shows that no compounds other than hydrocortisone were detected in the suspension used for injection.

The 4 post-injection chromatograms, run on 5 cm papers, revealed chiefly unchanged hydrocortisone. Additional weak reducing bands indicated metabolic products of hydrocortisone retaining the α-ketol structure. In order to concentrate these materials, all sections of each of the 4 papers above the hydrocortisone band were eluted and combined. Eluates of the four 5-hour papers containing the faster moving compounds were also combined.

Fig. 2 shows the results obtained when the eluates of these combined cuts were rechromatographed on narrower strips. Three distinct bands were found. The slow moving material found above the hydrocortisone band yielded a band which had scarcely moved after 7 days. The combined eluates of the previous 5-hour papers yielded 2 more bands, having mobilities similar to cortisone and corticosterone (compound B) respectively.

In Fig. 3 are shown the results of experiments in which cortisone acetate was injected intra-articularly. The extract of 130 ml of control fluid obtained from this subject (H. G.) was chromatographed on a 0.5 cm paper. Only one faint, slow moving band was seen. Chromatography of the injected suspension revealed the presence of free cortisone as well as the acetate. In the post-injection experiment, the synovial fluid was aspirated 2 hours after the injection of 100 mg of cortisone acetate. After extraction and chromatography on a one cm paper for 5 days, a strong band was seen above the free cortisone. The mobility of this material was similar to that of tetrahydrocortisone. The sulfuric acid chromogen absorption curve (10) of this band was similar to that of tetrahydro cortisone and definitely indicated the absence of hydrocortisone in this band. On reapplying the runoff from the 5-day paper for a period of 5 hours, 2 weak bands moving faster than the cortisone acetate were seen.

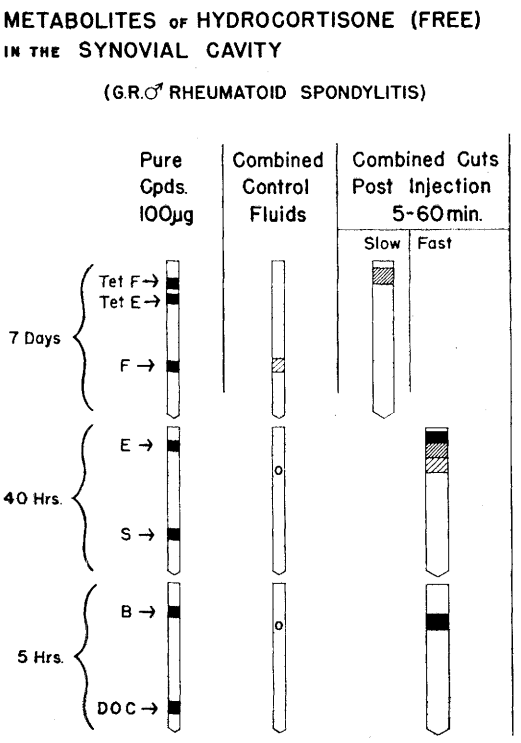

FIG. 2. Metabolites of hydrocortisone (free) in the synovial cavity. "Tet F" is tetrahydro-hydrocortisone; "Tet E" is tetra-hydrocortisone; "F" is hydrocortisone; "E" is cortisone; "S" is 11-desoxy-17-hydroxy corticosterone; "B" is corticosterone; "DOC" is 11-desoxycorticosterone.

Five minute and one hour experiments (Fig. 3) were performed on another subject (G. R.), after the injection of 50 mg of cortisone acetate. When these extracts were chromatographed, no new bands were seen after 5 minutes in the knee, while material moving more slowly than cortisone was seen after one hour.

Discussion. The apparent rapid disappearance of the relatively insoluble corticosteroids, cortisone and hydrocortisone, from the synovial fluid was quite surprising. It is possible that the low recoveries after injection are due in part to the mechanical inaccessibility of a portion of the injected material. When cortisone acetate suspension was injected into synovial fluid contained in a cellophane bag, however, mechanical settling occurred too slowly to account for the extremely low recoveries after one and 3 hours. It seems likely, therefore, that a definite adsorption or absorption process involving the synovial tissue takes part in the removal of injected steroid from the synovial fluid. Since, as indicated by our results, hydrocortisone and cortisone are removed from the synovial fluid at essentially the same rate, the difference in their therapeutic activity locally cannot be ascribed to a difference in ease of absorption by the synovial membrane.

Chromatographic analysis of the post-injection extracts indicates the presence of metabolites of the compounds originally injected. Conclusive identification of these metabolites must await the accumulation of sufficient material to yield more positive information than that afforded by spot tests on the paper chromatograms. These experiments are now in progress. The appearance of well localized bands which were not seen in either the control synovial fluid or in the suspension which was injected, are taken as strong evidence, however, that chemical transformation of the steroid compounds has been effected either by the synovial tissue, or by the inflammatory cells infiltrating the synovial membrane as a result of the arthritic process. It is believed probable that the metabolites found in synovial fluid represent the back diffusion of a small proportion of the new compounds being formed.

The finding of metabolites, in this instance, implies that the target tissue has metabolized the hormone which is acting upon it. On the basis of the present data it cannot, of course, be decided whether or not such transformations are essential to the therapeutic activity of the hormone. There is some suggestion, however, that hydrocortisone and cortisone are metabolized to different products, at least in part. The strongest bands in the hydrocortisone experiments were faster moving than hydrocortisone, whereas after cortisone injection the most abundant material had a slow mobility similar to that of tetrahydrocortisone.

Proof of intra-articular metabolism of steroid compounds requires elimination of the possibility that the metabolites which were observed had actually been formed elsewhere, as in the liver, for instance, and had returned to the synovial fluid from the general circulation, following absorption of the injected com-

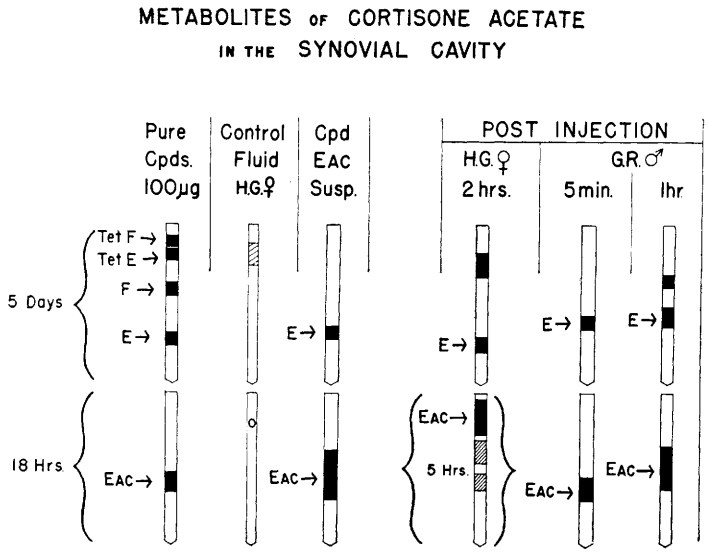

FIG. 3. Metabolites of cortisone acetate in the synovial cavity. "Tet F" is tetrahydro-hydrocortisone; "Tet E" is tetrahydrocortisone; "E" is cortisone; "E_{AC}" is cortisone acetate.

pound from the joint. This possibility was investigated in an experiment in which 50 mg of hydrocortisone acetate were injected into one knee (Subject G. R.). Six hours later this knee was aspirated and fluid also removed at the same time from the contralateral joint. The chromatograms of fluid from the contralateral joint gave no evidence of any steroid whatsoever, while the fluid from the injected knee showed three definite bands in addition to the unchanged hydrocortisone acetate.

Further experiments now under way are directed toward more definite characterization and comparison of the intra-articular metabolites of hydrocortisone in both rheumatoid arthritis and other arthritides.

Summary. Quantitative assays have been performed on extracts of synovial fluid aspirated from patients with rheumatoid arthritis, following intra-articular injection of cortisone, hydrocortisone, and their acetates. After one hour and 3 hours about 85% and 98% respectively had disappeared. There was no marked difference in rate of disappearance among the 4 compounds. Paper chromatographic analyses of post-injection extracts revealed well localized bands which were not seen in the control fluid from uninjected joints, indicating that metabolites were formed within the synovial cavity.

1. Ziff, M., Scull, E., Ford, D., McEwen, C., and Bunim, J. J., *A. M. A. Arch. Int. Med.*, 1952, v90, 774.

2. Hollander, J. L., Brown, E. M., Jr., Jessar, R. A., and Brown, C. Y., *J.A.M.A.*, 1951, v147, 1629.

3. Stevenson, C. R., Zuckner, J., and Freyberg, R. H., *Ann. Rheum. Dis.*, 1952, v11, 112.

4. Porter, C. C., and Silber, R. H., *J. Biol. Chem.*, 1950, v185, 201.

5. Lowenstein, B. E., Corcoran, A. C., and Page, I. H., *Endocrinol.*, 1946, v39, 82.

6. Heard, R. D. H., and Sobel, H., *J. Biol. Chem.*, 1946, v165, 687.

7. Burton, R. B., Zaffaroni, A., and Keutmann, E. H., *J. Biol. Chem.*, 1951, v188, 763.

8. Zaffaroni, A., and Burton, R. B., *J. Biol. Chem.*, 1951, v193, 749.

9. Manaro, J. M., and Zygmuntowicz, A., *Endocrinol.*, 1951, v48, 114.

10. Zaffaroni, A., *J. Am. Chem. Soc.*, 1950, v72, 3828.

Received June 25, 1953. P.S.E.B.M., 1953, v83.

ERRATUM

Article 20202, Embryonic Development in Turkey Eggs. . . , by M. W. Olsen and S. J. Marsden, in the PROCEEDINGS, April, 1953, page 641, 2nd column, line 8 should read ". . . experiments are *now* being conducted at the U. S. Agricultural Research Center, Beltsville, Md."