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ASSESSMENT OF THE TOXICITY OF DELIVERY SYSTEMS FOR INJECTABLE CONTRACEPTIVES

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SUMMARY

A favored approach to sustained release of contraceptive drugs has been to incorporate them into polymers that undergo gradual hydrolysis. Assessment of the safety of such delivery systems when used for parenteral delivery of drugs must take account of both local effects and effects at a distance mediated by the products of hydrolysis. Many of the problems of such an assessment are identical with those presented by any "drug" substance and similar approaches are appropriate. Special aspects are associated with the physical damage that may be caused by implant masses, with the carcinogenic potential of impervious materials of large physical size, with the fact that cells at the site of injection may be exposed to disproportionately high concentrations of breakdown products for a long period of time, with possible antigenicity of macro-molecular materials, and with the fact that partial hydrolysis may release a series of fragments of differing molecular weights.

INTRODUCTION

A variety of delivery systems have been used and suggested for prolonging the release of injectable contraceptives. Among them have been pellets of steroids [1, 2], crystals of steroids selected for low solubility and delivered intramuscularly in suspension [3, 4], polymer capsules containing steroids which diffuse slowly through the capsule wall [5, 6], biodegradable matrixes with embedded drug which release the drug by hydrolysis of the matrix and by diffusion of the drug [7], and the polymers made by cross-linking steroids from which steroid is released by hydrolysis of the cross links [8, 9]. Many of the problems of toxicity assessment of the delivery systems are common to the assessment of toxicity of any material. A few are less universal. Perhaps the most important is the fact that the immediate area at the injection site is exposed to a uniquely high concentration of drug delivery system degradation products, and mechanical effects continuously over periods which may extend to several years. Another problem of the use of polymers as carriers is the possible release of a variety of degradation products of varying molecular weight and of possibly different toxicity. The present paper reviews approaches to these problems and presents some preliminary results on biodegradable delivery systems.

EXPERIMENTAL

Doses for implantation

Starch discs were prepared by freeze drying a 10% potato starch gel and compressing into pellets of 13 × 1·2 mm at 2000 lbs. pressure.

Collagen discs were prepared by Princeton Biomedix, Inc., Princeton, New Jersey [10] using ficin-treated beef tendon collagen. A dilute gel in 1% acetic acid was precipitated by dialyzing against ammonia solution then against water. The precipitate was filtered and the filter cake dried between porcelain discs at 30°C. Formaldehyde tanning was conducted by suspending discs cut from the cake for 24 h in 2% formaldehyde at pH 8. Glutaraldehyde tanning was done similarly. After tanning, the discs (12 × 0·47 mm) were washed 24 h in running water to remove aldehydes.

Polylactic acid was prepared by Research Triangle Institute, Research Triangle Park, N.C. [11] by heating the DL-lactide at 130°C *in vacuo* for 260 h with stannous octoate catalyst. The polymer was dissolved in acetone and precipitated with methanol and immediately washed with water in a blendor. The viscosity in benzene was 1·17 dl/g. Films were compression molded. Discs of 10 × 0·30 mm were cut from the films.

Polylactic acid of 0·40 dl/g was made [11] by degrading the high molecular weight material by dissolution in methylene chloride containing 10% methanol for 60 h.

The copolymer of lactic and polylactic acids was made by methods similar to those used for the polylactic acid. It contained 20 to 24 mol % glycolic ester groups. Its intrinsic viscosity in benzene was 0·39 dl/g.

The Silastic® rubber was prepared from Dow Corning Medical Grade Elastomer No. 382 and Dow Corning stannous octoate catalyst and cast on a glass plate. The preparation was allowed to cure several days before use. Discs were 1·2 × 0·7 mm.

L-Polyleucine of molecular weight 4440 was purchased from Sigma Chemicals and compressed into discs. Discs were 10 × 0·7 mm.

Implantation

Discs of test materials were implanted subdermally in female rats of about 200 g weight using sterile

techniques. The implantations were placed on the sides of the rats posterior to the rib cage. An incision was made on the opposite side as a control. Collagen and starch implants were sterilized by gamma radiation—other materials by 70% alcohol.

Determination of biodegradation

To increase the sensitivity of detection of biodegradation, special implants of polylactic and lactic-glycolic acid copolymers were used. Films of 10 μm thickness were used to cover both sides of a polyethylene support film. The weight of the films was known with accuracy. After recovery from animals, the films were hydrolyzed with base and the lactic acid content determined colorimetrically [12].

RESULTS AND DISCUSSION

Local tissue reactions

The assessment of local effects of implanted materials has received increasing attention in recent years as a result of the use of artificial organs, new dental materials, and new suture materials. The usual approach is to introduce the material into appropriate tissues and sacrifice animals at intervals to allow examination of the implant site for tissue change. Observations that can be made on the whole animal include edema, erythema, abcess formation, signs of tenderness and, in severe reactions, ulceration. Microscopically, cellular aggregation around the implant will be observed almost invariably and the amount, kind, and duration of aggregation is an indicator of the severity of the reaction. Polymorphonuclear leucocytes are usually particularly prominent early in the reaction with fibroblasts and lymphocytes following and macrophages becoming more prominent in a few days. Very early appearance of macrophages indicates a severe reaction and frequently presages later necrosis [13]. As repair proceeds, the fibroblasts become aligned and organized, and develop into a collagenous envelope. Macrophages continue to be prominent in chronic inflammation and giant cells may also appear. Other changes that can be observed microscopically may include edema, hemorrhage, necrosis, fatty infiltration, and calcification.

In our examination [7, 14] of candidate biodegradable carriers, we have looked at local reactions caused by a variety of starches; starches cross-linked with glutaraldehyde; collagen cross-linked with formaldehyde, glutaraldehyde, ascorbic acid, chloroperbenzoic acid, and irradiation treatment; polylactic acid, lactic-glycolic acid copolymer and polyleucine. When implanted subdermally in rats, the various starches caused greater tissue reaction than the collagen derivatives or lactic acid polymers. This was true both initially and at 30 days. By 30 days, discs of 1·2 mm thickness of the various starches had undergone nearly complete absorption. Blends made by combining starch and collagen or gelatin and tanning with formaldehyde lasted for over 180 days when implanted subdermally. They did, however, cause more tissue reaction both initially and later than collagen alone tanned with formaldehyde. The greater reaction was manifest in transient edema and persistent macrophages and giant cells. Even so, the reaction would be classified as mild with no signs of necrosis, hemorrhage, or calcification.

Comparisons of the tissue reactivity and estimates of the time of degradation of discs of 0·5 mm thickness are given in Table 1 for several biodegradable materials and Silastic®. All comparisons are based on subdermal implants in rats. For the polylactic polymer, the estimates of time for degradation of discs of 0·5 mm thickness derive from recovery of residues and assay [12] of lactic acid obtained after hydrolysis. The estimates obviously represent great extrapolations and may be in error, particularly since Cutright and Hunsuck[15] have noted that polylactic acid sutures seemed to show markedly increased rates of disappearance after 8 to 12 months. The estimates of breakdown time of collagen discs are based on recovery, drying and weighing, with the obvious errors associated with this technique. The level of tissue reaction was minimum in all instances so that gradations between them represent small differences. Starch showed the most severe reactions of the group. Collagen and Silastic® rubber showed more severe reactions at 1 week than at 1 month. Collagen tanned with formaldehyde showed a more pronounced reaction at one month than at one week. In other trials [10], the reaction at 6 months with collagen

Table 1. Relative rate of breakdown and relative tissue reaction of several biodegradable materials and Silastic®

Material	Predicted life of 0·5 mm thick disc-years	Relative Tissue reaction* 7 days	30 days	90 days
Starch	0·05	3	2	—†
Collagen	0·02	2	1	1
Collagen-HCHO	1	1	2	1
Collagen-glutaraldehyde	2	1	1·5	1
DL Polylactic acid-mol wt. 130,000	50	<1	<1	1
DL Polylactic acid-mol wt. 30,000	10	—†	—†	—†
Lactic-glycolic acid copolymer	5	<1	<1	—†
Silastic	—	1·5	<1	<1

* Graded on a scale from 0 to 4 with 0 representing no reaction and 4 representing severe reaction.
† Microscopic examination not done.

tanned with formaldehyde has been observed to be mild. As would be expected, the reaction zones became more organized and fibrous with time. This progression occurred more quickly with the less rapidly biodegradable materials. Preliminary results on polyleucine indicate a reaction similar to collagen.

Biodegradable polylactate and polyglycolate sutures have been the subject of extensive studies and polyglycolate sutures are distributed commercially [16]. Cutright, Beasley and Perez[17] compared the reactions caused by polylactic and polyglycolic acid sutures implanted intramuscularly in rats. They found the polyglycolate material to be the more rapidly degraded and to give the greater inflammatory reaction. The reaction to polylactic acid sutures was graded as mild to moderate at 5 days post-implantation and thereafter from mild to none. Some giant cells were evident throughout the 90 days of observation. Cutright, and co-workers [15, 18] have also investigated the use of polylactic acid sutures in repair of bone fractures in monkeys. The inflammatory reaction was mild and characterized by macrophages and giant cells.

Collagen has similarly been the subject of studies in its uses as a suture material and absorbable hemostatic sponge [19–22]. The observed severity of the inflammatory reaction depended on the species used for test and its timing depended on the degree of tanning [19]. The slower the rate of absorption, the more delayed and continued the inflammatory reaction [20]. The number of polymorphonuclear leucocytes was generally greater for the more rapidly absorbed materials [19].

The tissue reactions of a variety of other macromolecules have been observed and measured in considering their suitability in prosthetic devices [23–32].

Rapid estimation of local reactions

Several techniques for rapid assessment of toxic effects of macromolecules have been suggested. Hegyli[33] has used roller tube suspensions of organ slices from 8 to 14 days old chick embryos both to measure biodegradation and to observe for toxic effects on the cells. Hulbert, Klawitter and Bartles[34] have used implantation in the leg or wing muscle of the 18 days old chick embryo to give a rapid first assessment of effects on tissues. The chicks were sacrificed 11 days after hatching and the thickness of the cellular response noted. Homsy[35] has observed effects on tissue culture growth as a means of rapid assessment of effects on cells. He has heated plastics for 62 h at 115°C to simulate long exposure *in vivo* with consequent release of breakdown products before placing them in the tissue culture. He has found the amount of material that can be extracted by a simulated body fluid after such a treatment to be predictive of the effects on tissue culture.

As an aid to evaluation of local effects on tissues, Salthouse and Williganl[36] have used histochemical reactions which give indications of the enzymes released by degrading cells and lysosomes. They observed the presence of succinic dehydrogenase, acid phosphatase, amino peptidases and adenosine triphosphatase.

Factors affecting local tissue reactions

A variety of factors affect the observed inflammatory response and need to be taken into consideration in interpreting results and extrapolating to the human being. One such factor is the animal used for test. Variations in response between species are illustrated by the data from van Winkle, Sewell and Wiland[19] assembled in Table 2. Rigdon[28] using polyurethane and polyethylene saw a more vigorous response in mice than in rats and like van Winkle, Sewell and Wiland[19], saw a much less marked response in rabbits. Polymorphs were found in the implant vicinity (subcutaneous), for only a few days in rabbits and for at least 230 days in mice.

Within a species, there are relatively large differences in the response of individual animals. Differences of two to three fold in the thickness of the collagenous deposit formed around subdermal implants in rats have regularly been observed in our

Table 2. Absorption of plain catgut sutures by three species as reported by van Winkle, Sewell and Wiland (19)

Animal	Days for absorption	Reaction
Rat	25	*at* 5 *days*—edema, fibroplasia and polymorphs over wide area *at* 10 *days*—lymphocytes *later*—lymphocytes, mononuclear and giant cells
Rabbit	50	*Initial*—a few fibroblasts and some lymphocytes *later*—lymphocytes, mononuclear, occasional giant cells. Peak at 50% absorption.
Dog	>60 (50% at 60 days)	lymphocytes, mononuclear cells, fibroblasts and occasional polymorphs. Peak at about 15th day.

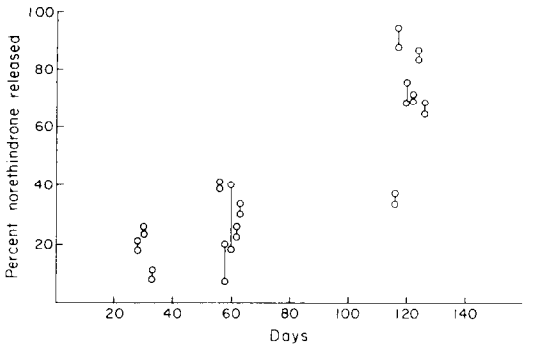

Fig. 1. Norethindrone released from formaldehyde tanned collagen discs with norethindrone dispersed throughout. Values for duplicate discs in a single rat are connected by a line.

studies. Differences in responses between implants when multiple implants are placed in the same animal are much less than the differences between animals. It is thought that these differences account for the differences between animals in steroid release from implants. In Fig. 1, the amount of norethindrone released from collagen implants is indicated [14]. Two implants were placed in each animal and the amount of steroid remaining determined after 30, 60, and 120 days. It is seen that the implants taken from a single animal have given more closely matched values for release than are seen among animals.

The site of implantation is of importance to the type and magnitude of reaction. Muscle implants frequently show greater reaction than subdermal implants, but in many cases the effect may be mediated mechanically. Tearing of fibers occurs when injections of solid materials are made in muscle, and Wood, Kaminski and Oglesby[37] noted that rods showed greater reaction than discs and that the reaction of discs was greatest at their edges. The effect of mechanical factors is also to be seen in subdermal implants. In our studies of collagen rods we saw severe reaction with rods of 3 cm. length placed subdermally in rats but very much less reaction with three rods of 1 cm. length.

The porosity of the surface of solid implants affects the reaction to them. As the porosity of the implant is increased, there is increased penetration of the cells associated with inflammatory reactions and formation of granulation tissue [29, 38]. Calcium deposition has been seen more frequently with implants of high porosity than with those of low porosity [29]. Tissue is likely to become attached to the rough porous surface, whereas the smooth-surfaced material is likely to become encased in a dense collagenous capsule.

Powders or crystals frequently give a more severe initial reaction but result in a more highly vascularized scar tissue. Shabad[39] studied films and minced films. The minced film caused an initially more severe inflammation with the individual particles quickly becoming separately encapsulated. The capsules remained thin as contrasted with those surrounding films of larger area. Powders that pack together *in vivo* may behave like films and produce a continuous envelope of low vasularity [40, 41].

Carcinogenicity

Assessment of the long term effects of injectable materials and their delivery systems presents special problems. Carcinogenic potential requires special attention because of the long exposure of a restricted site to what may be a high local concentration of the chemical breakdown products and to the mechanical effects of the drug and delivery system.

In the early 1940's, Turner[42] observed that Bakelite discs implanted subdermally in rats caused fibrosarcomas in a significant proportion of animals after 20 or more months. The phenomenon was extensively investigated by several groups of workers [40–54] with the finding that fibrosarcomas result from implantation of any of a variety of materials, including highly unreactive ones such as films or discs of Silastic®, polyethylene, platinum, glass, and solid aliphatic hydrocarbons. The number of tumors decreased with reduction in the size of the disc [40, 53] and decreased on perforating it [44]. Powders or textiles of the same materials generally did not result in tumor growth [44, 45]. Materials that caused marked inflammatory reaction were less tumorogenic than materials that caused lesser degrees of reaction [45]. The most tumorogenic materials seemed to cause the formation of a smooth, dense, but thin pocket [40, 45]. Characteristically it settled into a period of quiescence at 3 or 4 months with irregular cellular proliferation reappearing at 6 to 8 months after implantation in the rat [45]. It was, therefore, concluded that tumorogenesis was related to poor fluid exchange and reduced nutrition to surviving cells [40, 41]. An experiment that argues strongly against a chemical mediation was performed by Oppenheimer, Willhite, Danishevsky and Stout[54]. They implanted glass cover slips subcutaneously in the abdominal walls of rats and after 4 months removed them from some of the rats and used glass powder or polyethylene powder to fill the fibrous capsules that had formed around the cover slips. In other instances, glass or polyethylene powder was added to the capsule without removal of the cover slips. Tumors were formed in 20% of the instances in which the cover slip was left *in situ* and in only 2% of the cases in which it was replaced by glass or polyethylene powder. In another experiment, the same investigators [45] placed nylon fiber intradermally on one side of rats and nylon powder on the other side. Fifty five per cent of the rats developed tumors on the film side and none developed them on the powder side. Hueper[52] has vigorously disputed the theory that the tumors are caused by non-specific effects, resting his case on the variety of types of tumors seen after implantating a series of plastics at different sites in the rat, with some arising at sites distant from the site of implantation. The total incidence of tumors at sites distant from the site of implantation for animals with plastic im-

plants was, however, the same as total tumors in animals with no implants.

Tumors formed in response to implantation of solid materials of adequate size or of powders that give rise to dense, avascular, fibrous capsules have been observed in rats, mice and hamsters [52]. Stinson[53] obtained no tumors in guinea pigs in 30 months using materials that gave a 22% yield of tumors in rats. Cuadros and Brinson[55] have observed 22 monkeys with Silastic® implants of 2 cm. length and 0·24 cm. diameter for 6 years without encountering tumor generation.

Several observations bear on extrapolation of the rodent findings to human beings. One is that fibrosarcomas are relatively uncommon among neoplasms in the human being [56]. This is in spite of the fact that it is not uncommon that shell fragments and other foreign objects remain in tissues for many years. Bischoff[51] has assembled several case reports from the literature of tumors associated with foreign objects and has called attention [40, 41] to the fact that scars, and particularly burn scars, sometimes become the site of malignant neoplasms. It is to be noted, however, that by far the greater percentage of tumors associated with scars are carcinomas rather than fibrosarcomas [56–58].

In considering injectable delivery systems for drugs, caution dictates that they be kept as small as possible. If multiple units are used, they should not be placed so close together that their reactions will merge to produce a continuous collagenous barrier.

The assessment of the specific carcinogenic potential of injectable delivery systems presents the same problems as those of the assessment of the carcinogenicity of any drug. Exposure over most of the lifetime of the experimental animal and observation of tumor incidence as compared with suitable control groups is the only apparent approach. Because of the special problems of "solid state" tumors in rodents as discussed above, it is advisable to include groups with the carrier in powdered form in evaluating carcinogenicity in rodent species.

Systemic toxicity

The degradation of delivery systems can conceivably yield products of a variety of molecular weights and differing toxicity potential. The assessment of the hazard involved is not different from that of the assessment of the hazard of any drug or excipient candidate. The approaches that are accepted by common agreement and fiat are to test the material in several species over extended periods of time at multiples of the doses to be used in human beings looking for evidences of toxic effect by measuring indicators of organ function and by examining tissues histologically upon sacrifice of the animals. The fate of suitably labelled carrier should be followed to determine if there is evidence of accumulation in selected organs. If so, special attention must be given to the possibility of toxic effects in that organ. Degradation of labelled carrier in tissue culture systems coupled with suitable separation and isolation approaches will be useful in determining whether oligomer units are in fact released from the carrier or if the attack is at the end of chains so that only monomer units are released. Knowledge of the toxicity of the monomer is mandatory and it may be necessary to investigate the toxicity of oligomer units that prove to be principle products released systemically.

One reassuring factor is the very low dosage actually released daily. If an injectable that lasts 3 months includes 150 mg of carrier, the average quantity of carrier material released per day is less than 2 mg. If the monomer units are ubiquitous, non-toxic, biological materials such as amino acids or sugars or lactic acid, causes for concern are further reduced.

In evaluating toxicity and carcinogenicity, it must not be forgotten that the patient is exposed to the entire system, not just its principle ingredients. Residues of catalysts or added plasticizers or antioxidants are part of the total system. In the instance of local inflammatory responses, the magnitude of the reaction caused by plastics has in several cases been a function of placticizers and other low molecular weight additives [23, 32].

Effects on cellular components of blood and on blood clotting should receive at least the attention they would be given in the usual study of chronically administered drugs. The possibility of effects on blood components is, however, not of the same prime concern as with prosthetic devices inserted directly in the cardiovascular system.

Since some polymers reduce the friction of turbulent flow when present in concentrations of a few parts per million [59], the question can be raised of possible effects on blood rheology of polymer fragments from delivery systems. Significant effects seem unlikely since the effect is rapidly lost as the polymer chain is shortened and because the blood is already loaded with macromolecules.

Allergenic potential

The use of macromolecules as carriers raises special questions of allergenicity and hypersensitivity. The guinea pig is usually considered the best animal for testing sensitizing potential. Even so, it is less sensitizable than the human being [60]. Evaluation of allergenic potential should take account of both the possibility of circulating antibodies and the possibility of cellular immunity mediated by lymphocytes. Several methods are available for each but the low solubility of the biodegradable carriers will represent a problem in some cases. To test for circulating antibodies, the method-of-choice is probably that of sensitizing by exposing the guinea-pig to the carrier system placed intramuscularly or subdermally for 2 to 3 months and then conducting a complement fixation test [61] using the delivery system in subdivided form. To test for delayed hypersensitivity, the method-of-choice is probably that of collecting lymphocytes from the peritoneal cavity of animals exposed to the carrier material for a few days and for a few months, transferring

the lymphocytes to test animals and challenging 72 h later by injecting a small amount of the carrier material [62].

A maximum challenge of sensitizing potential and a technique which shortens the sensitizing period is to inject the test material together with Freunds' adjuvant [60, 25].

Van Winkle, Sewell and Wiland[19] attempted unsuccessfully to demonstrate antigenicity of collagen sutures by several techniques. These included injecting guinea-pigs with suspensions together with killed tubercle bacteria and later injecting suspensions of suture material i.v. to elicit anaphylaxis, exposure *in vitro* of the uterus of "sensitized" guinea-pigs to suspensions of sutures to elicit contractions, and implanting guinea-pigs with sutures after "sensitization" by the Freund–McDermott technique. Steffen and Timpl[63], however, did find evidence of circulating antibodies after injecting purified collagen in rabbits. Michaeli and coworkers[64] successfully produced antibodies in rabbits by injecting guinea-pig collagen. Synthetic polypeptides containing more than one amino acid may be antigenic but antigenicity of homopolymers of amino acids is rare or non-existant [65, 66]. In spite of failure to demonstrate sensitization to amino acid homopolymers, sera from "sensitized" animals has appeared to interact *in vitro* with a variety of blood proteins. Maurer[67] has voiced conviction that such apparent interactions are artifacts.

Fate of delivery system components

The rate of completeness of disappearance of biodegradable carriers must be known as part of the evaluation of their potential for causing toxic manifestations. Labelling of the carrier and collection of excreta and, if necessary, expired gases yields information on the rate at which is it totally cleared from the body and allows examination of the distribution of intermediate products of its metabolism within the body. Kulkarini *et al.*[68] tagged polylactic acid in the α-carbon and measured radioactivity appearing in feces, urine, and tissues. They found no significant radioactivity in any of these sites and concluded that oxidation and elimination of radioactivity in CO_2 must be occurring. Recovery of the implants showed 14% to have been lost in 3 months. Brady *et al.*[69] confirmed that only negligible accumulation of radioactivity from labelled polylactate could be found in tissues and showed the disappearance of label from the implant site to be at a constant rate for at least 168 days in rats. Wade and Leonard[70] have similarly followed the excretion of tagged polymethyl-2-cyanoacrylate.

A variety of systems have been used for rapid estimation of the rate of degradation and for comparing relative rates of degradation of polymers. Kulkarini and coworkers[71] measured degradation of polylactic acid by dispersing labelled polylactic acid in chick embryo liver homogenates and determining the increase in radioactivity of the centrifuged filtrate as a function of time. Hegyli[33] measured breakdown in a similar manner using organs of 8 to 14 days old chick embryos in roller-tube cultures.

In extrapolating rates of breakdown to conditions of actual use, it is important to keep in mind a number of precautions. One is that the geometry of the unit may have profound effects on the rate of breakdown. Since attack will be from the surface, the first approximation is that the degradation will be to a constant depth per unit time. This may not, however, be true. As the surface becomes roughened through loss of medicament and some degree non-uniform attack, phagocytosis of projecting fragments may hasten their breakdown. In the case of polylactic acid, some evidence of increasing rates with time have been seen [15]. These effects might be autocatalytic in nature. The geometry will also affect the amount of tissue irritation. This will have two opposing effects. One will be to increase the migration of cells associated with inflammatory reaction and their contributions of increased enzymatic activity in the area. The other will be to increase the walling off of the injected material and to lessen vascularity. This would be expected to decrease the rate of breakdown. Some reflections of these factors must account for the differences in release of steroid from equivalent implants in different individual animals shown in Fig. 1.

Another precaution is that of making certain of replicability of polymer characteristics. Experience with polylactic acid shows the breakdown rate to be considerably affected by molecular weight. This is reflected in Table 1.

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REFERENCES

1. Deanesly R. and Parker A. S.: *Lancet* **ii** (1938) 606–608.
2. Perloff W. H.: *J. clin. Endocr. Metab.* **10** (1950) 447–454.
3. Helmreich M. L. and Huseby R. A.: *Steroids suppl* **2** (1965) 79–95.
4. Zanartu J. and Navarro C.: *Obstet. Gynec.* **31** (1968) 627–633.
5. Dziuk P. J. and Cook B.: *Endocrinology* **78** (1966) 208–211.
6. Segal S. J. and Croxatto H.: Presented at 23rd annual meeting of American Fertility Society, Washington, D. C., April 14–16, (1967).
7. Jackanicz T. M., Nash H. A., Wise D. L. and Gregory J. B.: *Contraception* **8** (1973) 227–234.
8. Diczfalusy E.: *Endocrinology* **54** (1954) 471–476.
9. Kuhl H. and Tabuert H. D.: *Steroids* **22** (1973) 73–87.
10. Bulbenko G. F. and Hollis R. A.: unpublished results.
11. Schindler A.: unpublished results.
12. Barker S. B.: In *Methods in Enzymology* (Edited by S. P. Colwick and N. O. Kaplan). Academic Press, New York, Vol. III (1957) 241–246.
13. Turner J. E., Lawrence W. H. and Autin J.: *J. biomed. Mater. Res.* **7** (1973) 39–58.

14. Jackanicz T. M. and Sederholt D.: unpublished results.
15. Cutright D. E. and Hunsuck E. E.: *Oral Surg.* **33** (1972) 28–34.
16. Anscombe A. R., Hira N. and Hunt B.: *Brit. J. Surg.* **57** (1970) 917–920.
17. Cutright D. E., Beasley J. D. and Perez B.: *Oral Surg.* **32** (1971) 165–173.
18. Cutright D. E., Hunsuck E. E. and Beasley J. D.: *J. oral Surg.* **29** (1971) 393–397.
19. Van Winkle W., Sewell W. and Wiland J.: *J. Am. Geriat. Soc.* **3** (1955) 572–579.
20. Rudenstam C. M. and Zederfeldt B.: *Acta clin. scand.* **134** (1968) 503–509.
21. Salthouse T. N. and Williams J. A.: *J. surg. Res.* **9** (1969) 481–486.
22. Choapil M. and Holusa R.: *J. biomed. Mater. Res.* **2** (1968) 245–264.
23. Autin J.: *J. biomed. Mater. Res.* **1** (1967) 433–449.
24. Turner J. E., Lawrence W. H. and Autin J.: *J. biomed. Mater. Res.* **7** (1973) 39–58.
25. Lawrence W. H., Malik M. and Autin J.: *J. biomed. Mater. Res.* **8** (1974) 11–34.
26. Imai Y., von Bally K. and Nosé, Y.: *Trans. Am. Soc. artif. Org.* **16** (1970) 17–25.
27. Rigdon R. H.: *J. biomed. Mater. Res.* **7** (1973) 79–93.
28. Rigdon R. H.: *J. biomed. Mater. Res.* **8** (1974) 97–117.
29. Šprincl L., Vacik J. and Kopečik J.: *J. biomed. Mater. Res.* **7** (1973) 123–136.
30. Ulbrich K., Šprincl L. and Kopečik J.: *J. biomed. Mater. Res.* **8** (1974) 155–161.
31. Kopeček J., Šprincl L., Bažilova H. and Vacik J.: *J. biomed. Mater. Res.* **7** (1973) 111–121.
32. Guess W. L. and Haberman S.: *J. biomed. Mater. Res.* **2** (1974) 313–335.
33. Hegyeli A. F.: *J. biomed. Mater. Res.* **7** (1973) 205–214.
34. Hulbert S. F., Klawitter J. J. and Bartles D. M.: *J. biomed. Mater. Res.* **8** (1974) 137–153.
35. Homsy C. A.: *J. biomed. Mater. Res.* **4** (1970) 341–356.
36. Salthouse T. N. and Willigan D. A.: *J. biomed. Mater. Res.* **6** (1972) 105–113.
37. Wood N. K., Kaminski E. J. and Oglesby R. J.: *J. biomed. Mater. Res.* **4** (1970) 1–2.
38. Šprincl L., Kopečik J. and Lím D.: *J. biomed. Mater. Res.* **5** (1971) 447–458.
39. Shabad L. M.: In *Potential Carcinogenic Hazard of Drugs* (Edited by R. Truhaut). Springer-Verlag, New York (1967) 188–195.
40. Bryson G. and Bischoff F.: *Progress in Experimental Tumor Research* (Edited by F. Homberger) **9** (1967) 77–164.
41. Bischoff F. and Bryson G.: In *Progress in Experimental Tumour Research* (Edited by F. Homburger) **5** (1964) 85–133.
42. Turner F. C.: *J. natn. Cancer Inst.* **2** (1941) 81–83.
43. Oppenheimer B. S., Oppenheimer E. T., Stout A. P. and Danishefsky I.: *Science* **118** (1953) 305–306.
44. Oppenheimer B. S., Oppenheimer E. T., Danishefsky I., Stout P. and Erich F. R.: *Cancer Res.* **15** (1955) 333–340.
45. Oppenheimer B. S., Oppenheimer E. T., Stout A. P., Willhite M. and Danishefsky I.: *Cancer* **11** (1958) 204–213.
46. Oppenheimer B. S., Oppenheimer E. T. and Stout A. P.: *Proc. Soc. exp. Biol. Med.* **79** (1952) 366–369.
47. Northdurft H.: *Naturwissenschaften* **42** (1955) 106.
48. Northdurft H.: *Naturwissenschaften* **42** (1955) 75–76.
49. Bischoff F.: In *Advances in Lipid Research* (Edited by R. Paoletti and D. Kritchevsky). Academic Press, New York, Vol. 7 (1969) 166–244.
50. Bryson G. and Bischoff F.: In *Progress in Experimental Tumor Research* (Edited by F. Homberger). **11** (1969) 100–133.
51. Bischoff F.: *Clin. Chem.* **18** (1972) 869–894.
52. Hueper W. C.: *Path Microbiol.* **24** (1961) 77–106.
53. Stinson N. E.: *Brit. J. exp. Path.* **45** (1964) 21–29.
54. Oppenheimer E. T., Willhite M., Danishevsky I. and Stout A. P.: *Cancer Res.* **21** (1961) 132–134.
55. Cuadros A. and Brinson A. O.: unpublished results.
56. Kanaar P. and Oort J.: *Dermatologia* **138** (1969) 312–319.
57. David M.: *Plastic reconstr. Surg.* **38** (1966) 429–437.
58. Arons M. S., Lynch J. B., Lewis S. R. and Blocken T. G.: *Ann. Surg.* **161** (1965) 170–188.
59. Kenis P. R.: *J. appl. Polym. Res.* **15** (1971) 607–618.
60. Magnusson B. and Kligman H. M.: *J. invest. Derm.* **52** (1969) 268–276.
61. Kwapinski J. B. L.: In *Methodology of Immunol. Chemical and Immunol. Res.* Wiley Interscience, New York (1972) 482–525.
62. Campbell D. H., Garvey J. S., Cremer N. E. and Sussdorf D. H.: In *Methods in Immunology* (Edited by W. A. Benjamin) 2nd ed. New York (1970) pp. 000–000.
63. Steffen C. and Timpl R.: *Int. Arch. Allergy* **22** (1963) 333–349.
64. Michaeli D., Benjamin E., Leung D. Y. K. and Martin D. R.: *Immunology* **8** (1971) 1–6.
65. Sela M.: In *Advances in Immunology* (Edited by F. J. Dixon, Jr. and J. H. Humphrey). Academic Press, New York, Vol. 5 (1966) pp. 29–129.
66. Maurer P. H.: In *Progress of Allergy* (Edited by P. Kallos and B. H. Waksman). S. Karger, New York (1964) pp. 1–40.
67. Maurer, P. H., Subrahmanyani D., Katchalski E. and Blout E. R.: *J. Immunol.* **83** (1959) 193–197.
68. Kulkarni R. K., Pani K. C., Neuman C. and Leonard F.: *Archs Surg.* **93** (1966) 839–843.
69. Brady J. M., Cutright D. E., Miller R. A. and Battistone G. C.: *J. biomed. Mater. Res.* **7** (1973) 155–166.
70. Wade C. W. R. and Leonard F.: *J. biomed. Mater. Res.* **6** (1972) 215–220.
71. Kulkarni R. R., Moore E. G., Hegyli A. F. and Leonard F.: *J. biomed. Mater. Res.* **5** (1971) 169–181.